Proceedings
of the
Society
for
Experimental Biology and Medicine

VOL. 92 MAY, 1956 No. 1

SECTION MEETINGS

DISTRICT OF COLUMBIA	
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NEW YORK	
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Zone Electrophoresis in Studies of Maternal and Fetal Sera.* (22365)

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Qualitative and quantitative differences between concentrations of serum proteins in fetal, maternal and normal bloods have been demonstrated by means of electrophoresis(1, 2) and other fractionation methods(3,4). Differences between maternal and fetal serum polysaccharide levels have also been observed (5). The purpose of this paper is to describe a qualitative study of serum proteins and polysaccharides in sera from 15 pregnant women and their newborn offspring using the technic of zone electrophoresis.

Materials and methods. Fifteen normal women in the third trimester of pregnancy were selected for study. Blood samples were collected from one week to 3 months before delivery and again at the time of parturition. A sample of uncontaminated fetal blood was obtained immediately after birth of the child from the umbilical vein of the cord on the placental side after clamping and cutting the cord. Filter paper electrophoresis was carried out using the technic of Kunkel and Tiselius(6). Each experiment was usually performed on duplicate sheets of filter paper using technics which have been described elsewhere(7,8). Following electrophoresis of the proteins, staining was carried out using an alcoholic mixture of mercuric chloride and brom phenol blue(6). Protein-bound polysaccharides were stained with basic fuchsin reagent following fixation and treatment with periodic acid(9-11).

Results. All of the electrophoretically separated maternal sera stained for proteins showed a decrease in albumin and gamma globulin and an increase in the beta and alpha globulins as compared with normal controls.

* The authors are grateful to Dr. John Weber of Pittsburgh, Pa. for making patients available and to Dr. John Gruhn, St. Joseph's Hospital, Pittsburgh, Pa. for obtaining many of the blood specimens. The Figures were prepared by Mrs. Barbara Davidson.

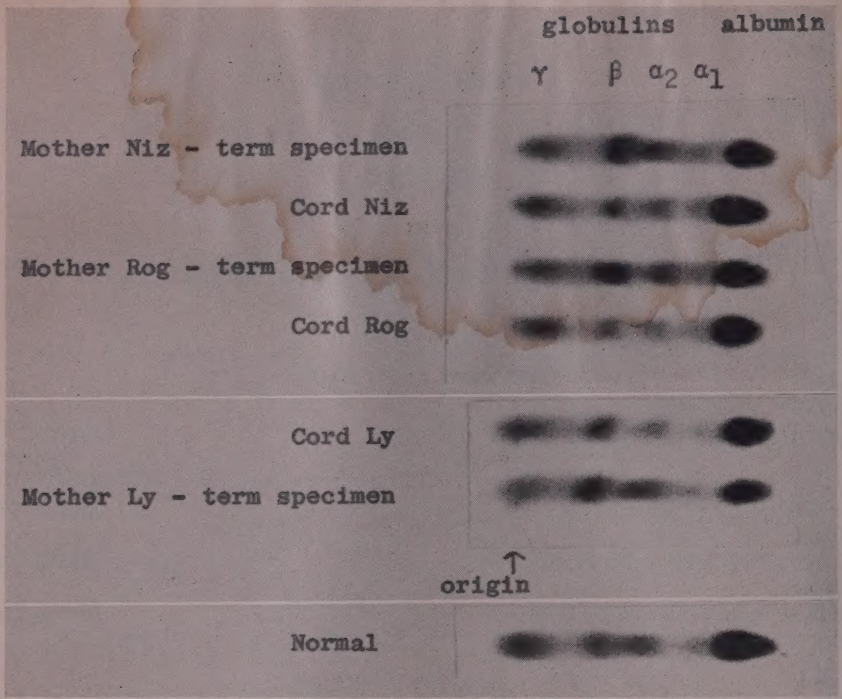

FIG. 1. Comparisons between zone electrophoresis patterns of maternal and cord sera stained for proteins.

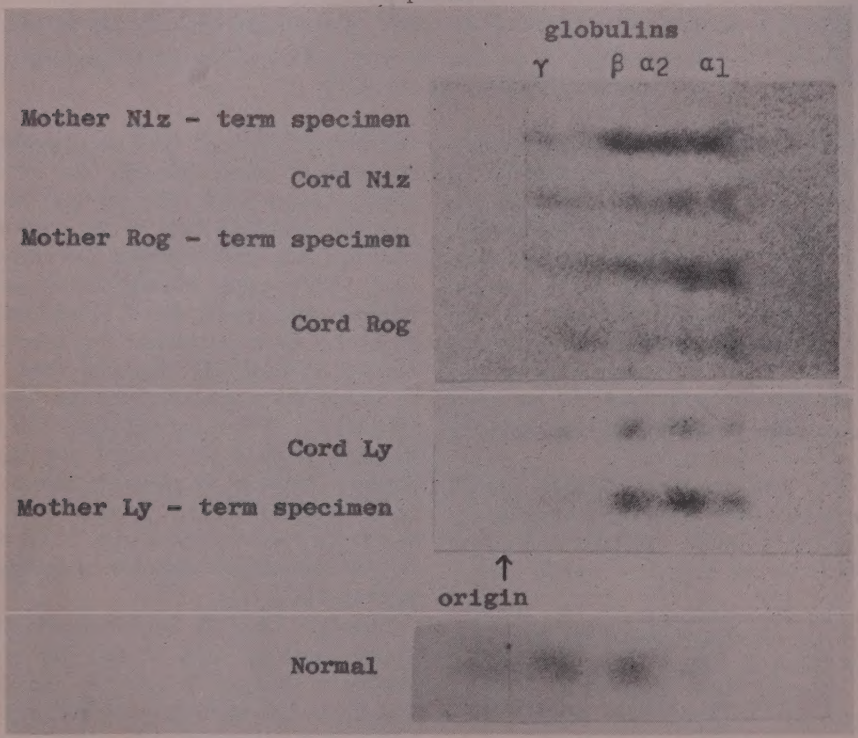

FIG. 2. Comparisons between zone electrophoresis patterns of maternal and cord sera stained for protein-bound polysaccharides.

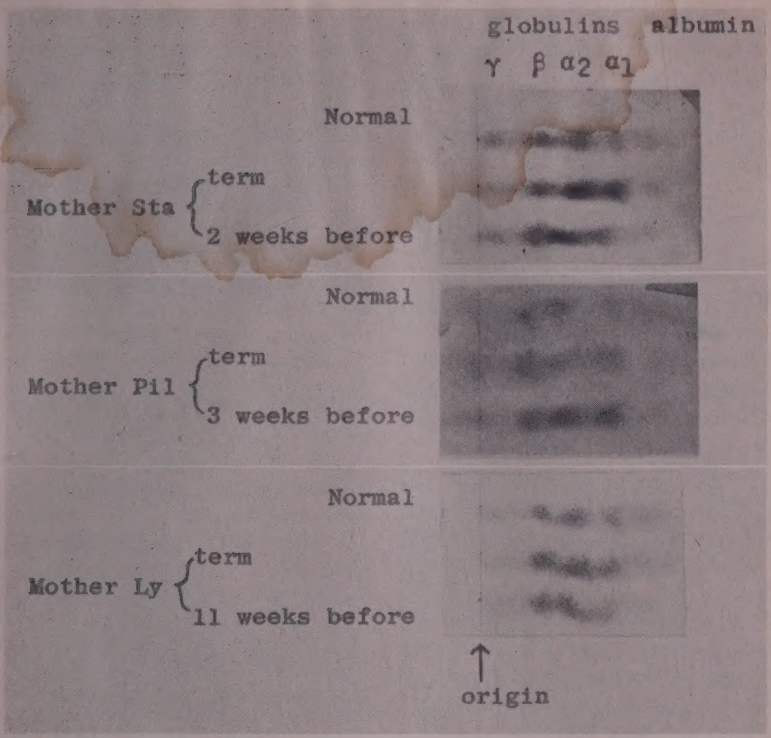

FIG. 3. Protein-bound polysaccharides in electrophoretically separated maternal sera obtained during last trimester of pregnancy and at term.

Corresponding cord sera which were stained for proteins showed relatively normal albumin patterns but decreases in total globulin. Alpha and beta globulins were decreased as compared with corresponding maternal patterns and also with normal patterns. Gamma globulin in cord blood specimens showed an increase above the maternal gamma globulin. Protein patterns of three maternal-cord serum combinations are shown in Fig. 1.

Differences in the electrophoretic distribution of protein-bound polysaccharides were also observed between maternal and cord sera. There were marked increases in protein-bound polysaccharides in all maternal sera. This was frequently reflected uniformly throughout the $alpha_1$, $alpha_2$ and beta globulins. On the other hand, the protein-bound polysaccharides contained in corresponding cord sera usually showed decreases as compared with normal patterns. The component associated with gamma globulin seemed to be about the same in maternal and cord sera and in normal sera (Fig. 2).

In 5 instances more than one pre-term serum specimen was studied for polysaccharides. The results of this study indicated that these components increased in two patients decreased in one patient, and remained the same in two patients during the last trimester of pregnancy. Comparative patterns of sera from three persons are illustrated in Fig. 3.

Discussion. The present work provides qualitative confirmation of previous electrophoresis studies which have shown that the concentrations of alpha and beta globulins in sera of pregnant women are significantly elevated above normal and that the concentrations of fetal alpha and beta globulins are lower than maternal values(1). On the other hand, the concentration of gamma globulin in fetal sera is higher than in maternal sera. Protein-bound polysaccharides determined by

the periodic-acid-Schiff stain showed considerable increases in maternal sera as compared with corresponding cord sera or normal sera. These findings parallel the glucosamine and non-glucosamine polysaccharide content of maternal and cord sera(5). This does not necessarily indicate that the same materials are being demonstrated by the different technics. The periodic-acid-Schiff stain yields positive results when materials contain vicinal OH or NH_2 groups which can be oxidized to aldehyde. In serum only the high molecular weight substances (*i.e.* protein-bound polysaccharides and glycoproteins) possessing these characteristics are likely to be present in adequate amounts to give visible color.

Summary. Zone electrophoresis was carried out on 15 pairs of maternal and cord sera. Qualitative differences between all maternal and cord bloods were observed in electrophoretic patterns stained for proteins and protein-bound polysaccharides. Maternal sera consistently showed decreases in albumin and gamma globulin and increases in alpha and beta globulins. Corresponding cord sera exhibited decreases in alpha and beta globulins and an increase in gamma globulin. Protein-bound polysaccharides associated with alpha and beta globulins were markedly increased in mothers' sera but were decreased below normal in the corresponding cord serum specimens.

1. Longsworth, L. G., Curtis, R. M., and Pembroke, R. H., *J. Clin. Invest.*, 1945, v24, 46.

2. Mack, H. G., *The Plasma Proteins in Pregnancy.* Charles Thomas, Springfield, Ill., 1955.

3. Plass, E. D., and Matthew, C. W., *Am. J. Obst. and Gynec.*, 1926, v12, 847.

4. Rapoport, M., Rubin, M. I., and Chaffee, D., *J. Clin. Invest.*, 1943, v22, 487.

5. Shetlar, M. R., Kelly, K. H., Foster, J. V., Shetlar, C. L., and Everett, M. R., *Am. J. Obst. and Gynec.*, 1950, v59, 1140.

6. Kunkel, H. G., and Tiselius, A., *J. Gen. Physiol.*, 1951, v35, 89.

7. Kuhns, W. J., and Crittenden, J., *J. Lab. and Clin. Med.*, 1955, v46, 398.

8. Kuhns, W. J., *Am. Pract.*, 1955, v6, 1157.

9. McManus, J. F. A., *Nature*, 1946, v158, 202.

10. Hotchkiss, R., *Arch. Biochem.*, 1948, v16, 131.

11. Köiw, S., and Grönwall, A., *Scand. J. Clin. and Lab. Invest.*, 1952, v4, 244. (Suppl).

Received March 15, 1956. P.S.E.B.M., 1956, v92.

C-Reactive Protein during Pregnancy and in Cord Blood. (22366)

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C-reactive protein (CRP) an abnormal constituent of human serum(1,2), is found in a variety of pathologic conditions(3,4). This protein, considered as a very sensitive index to inflammation or tissue destruction(5,6), has been crystallized and shown to be a beta globulin(7,8). CRP has been reported to be present in the late months of pregnancy (9,10).

The purpose of the present study was to examine the occurrence of CRP in a larger series of pregnant women and to see whether it passes the placental barrier. Since antistreptolysin O was found to occur in cord blood at a titer comparable to that of the maternal blood(11,12), determination of antistreptolysin O in maternal and foetal blood was included for purpose of comparison. CRP was also determined in a limited number of very young babies.

Materials and methods. Ninety-one women in the 2nd and 28 in the 3rd trimester of pregnancy together with 71 women in labour, were examined for the presence of CRP in their serum. Their ages varied from 19 to 42. Only healthy persons were included. Blood was taken during labour *i.e.* 2-12 hours before delivery and before the start of any necessary intervention. Concomitantly cord blood was also examined. As there were 2 pairs of

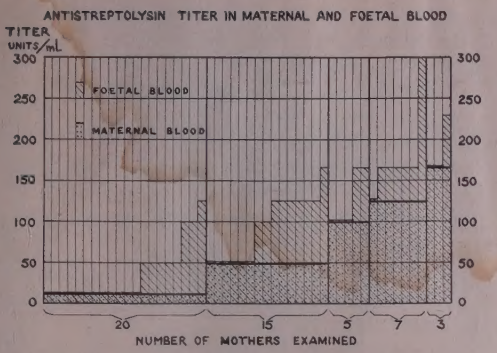

FIG. 1.

twins the number of cord samples examined reached 73. CRP determination was performed by the capillary precipitin method of Anderson and McCarty(13), employing commercial CRP antiserum.* Each millimeter of precipitate was considered as 1+. The degree of precipitation (1+ to 6+) was read after 2 hours incubation at 37°C and overnight at 6°C. Antistreptolysin O titer was tested in the sera of 50 of the above mentioned mothers and cords. The technic of the test was that described by Rantz and Randall(14). Bacto-Streptolysin O reagent was used.

Results. Results of the CRP tests are summarized in Table I.

TABLE I. C-Reactive Protein in Sera of Pregnant Women and in Cord Blood.

	2nd trimester of pregnancy	3rd trimester of pregnancy	During labor	Cord blood
No. of sera examined	91	28	71	73
No. positive	23	9	47	1

CRP was found in 25% of sera taken in the second trimester and in 32% from the third trimester of pregnancy. The percentage of positives was 66% during labour. Of the 47 positive sera, 24 gave 1+, 9 gave 2+, and 11 gave 3+ to 5+. The results were not influenced by age or number of pregnancies. Of 73 samples of cord blood only one gave a positive (2+) reaction. In this case labour lasted 48 hours and an amniotic infection occurred. The baby was delivered by forceps and developed conjunctivitis after birth. Maternal blood taken before delivery gave a 2+ result.

Antistreptolysin O was present in all 50 cord bloods examined. Fig. 1 shows that the antistreptolysin O titer was the same as in maternal blood in 24 samples and even higher in 26, in some of these significantly so.

CRP in babies. A limited number of tests showed that CRP can be produced by very young babies. CRP in amounts of 1+ to 4+ was observed in a blood sample of a baby 2 days old suffering from pemphigus neonatorum, and in a blood sample of a baby 2 weeks old with staphylococcal pneumonia. Blood from the heart of 2 babies who died at the ages of 2 and 3 weeks from pneumonia, gave positive CRP reactions. CRP was also found in 2 pyrexial babies 5 and 6 months old.

Comment. CRP was found in the blood of women in the third trimester of pregnancy in the same percentage as by Shetlar *et al.*(10). Two-thirds of women developed CRP during labour. CRP levels run roughly parallel with erythrocyte sedimentation rate (ESR) in inflammatory processes(13), but there was no correlation between increase in the rate of sedimentation (Westergren) and degree of CRP positivity in the 71 women examined before delivery. The ESR was raised in 70 of them. The occurrence of CRP in only one of 73 cord samples examined, indicates that this protein does not pass the placental barrier. The absence of CRP even in cord bloods when maternal blood yielded 2+ to 5+ levels antepartum, was the more striking when compared with the high antistreptolysin O titer of the cords. In the one case in which CRP was demonstrated in foetal blood this protein was apparently formed by the foetus itself due to infection *in utero* during the protracted 48 hours labour. The occurrence of CRP in very young babies accords with the view that CRP is not an antibody (15,16) and its production does not require therefore the maturity of antibody forming mechanism.

Summary. 1. The sera of 119 pregnant

* Obtained from Schieffelin & Co., New York.

women and 71 women in labour were examined for C-reactive protein. 25% to 32% of the pregnant women and 66% of the women in labour showed positive results. 2. Only one of 73 cord bloods examined showed the presence of C-reactive protein. 3. Antistreptolysin O was present in all 50 cord bloods examined, frequently at a titer higher than that in maternal blood. 4. C-reactive protein was found in blood of very young babies suffering from bacterial infections. 5. It is suggested that C-reactive protein is not transferred across placental barrier.

1. Tillet, W. S., and Francis, T. Jr., *J. Exp. Med.*, 1930, v52, 561.

2. Abernethy, T. J., and Avery, O. T., *ibid.*, 1941, v73, 173.

3. Ash, R., *J. Inf. Dis.*, 1933, v53, 89.

4. Lofstrom, G., *Brit. J. Exp. Path.*, 1944, v25, 21.

5. McCarty, M., *Ann. Int. Med.*, 1952, v37, 1027.

6. Roantree, R. J., and Rantz, L. A., *Arch. Int. Med.*, 1955, v96, 674.

7. McCarty, M., *J. Exp. Med.*, 1947, v85, 491.

8. Wood, H. F., McCarty, M., and Slater, R. J., *ibid.*, 1954, v100, 71.

9. Muschel, L. H., and Weatherwax, R. J., PROC. SOC. EXP. BIOL. AND MED., 1954, v87, 191.

10. Shetlar, M. R., Bullock, J. A., Shetlar, C. L., and Payne, R. W., *ibid.*, 1955, v88, 107.

11. Plummer, H., *J. Immunol.*, 1938, v35, 235.

12. Vahlquist, B., Lagercrantz, R., and Nordbring, F., *Lancet*, 1950, vii, 851.

13. Anderson, H. C., and McCarty, M., *Am. J. Med.*, 1950, v8, 445.

14. Rantz, L. A., and Randall, E. A., PROC. SOC. EXP. BIOL. AND MED., 1945, v59, 22.

15. Good, R. A., *J. Lab. Clin. Med.*, 1954, v44, 803.

16. Wood, H. F., *J. Exp. Med.*, 1953, v98, 321.

Received March 27, 1956. P.S.E.B.M., 1956, v92.

Cytoautoradiography Fe^{59} Uptake by Bone Marrow Cells. I. Normal Human Erythroblasts and Young Erythrocytes.* (22367)

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In vivo Fe^{59} uptake was first demonstrated in rat bone marrow by stripping film cytoautoradiography of marrow smears after injection of considerable amounts of radioiron (1).

The present paper reports the *in vitro* uptake of Fe^{59} by normal human erythroblasts and young erythrocytes during incubation with small amounts of radioiron.

Method. Human bone marrow from normal and pathological subjects was obtained by sternal aspiration with oxalated or heparinized syringes. Samples of 0.5-1 ml marrow were quickly suspended in a buffered solution of Ferrous^{59} citrate containing 3-5 μc, specific activity 3.79 μc/μg Fe. The tubes were incubated at 37° for 1-2-4-6-9-12-24 hours, then centrifuged at low speed. The supernatant fluid was then discarded and the cells washed twice with normal saline solution. This procedure usually removed the excess radioiron but sometimes damaged the cells. The following alternative procedure was later adopted. The marrow-radioiron solution was put into vertical sedimentation rate pipettes and incubated at 37°. After the desired time, thin smears were prepared from the upper part of the cellular layer, where the marrow particles are easily recognized. With this procedure the Fe^{59} background obtained is only slightly higher than with the washing method. The smears, dried at room temperature and fixed with pure methanol, were treated with the usual stripping film pro-

* These researches were initiated at University of California, when author held a Fulbright-Smith-Mundt Fellowship, and were completed at Institute of Medical Pathology in Padua. The work done in the Donner Laboratory was partially supported by the Henry Stevens Kiersted Memorial Fund.

The author is grateful to Professor John H. Lawrence for his encouragement and assistance.

TABLE I. *In Vitro* Fe^{59}-Uptake by Normal Human Erythroblasts. Percent of *labeled* and *non-labeled* cells and their *activity* (expressed by addition of plusses of the marked erythroblasts of each stage) after incubation with 5 μc of Ferrous^{59} citrate $\left(\text{spec. activity } \frac{3.79\ \mu c}{\mu g\ Fe}\right)$.

Kodak permeable base stripping film (experimental) exposure: 17 d. Case 76: C. Corrado, 30-yr-old healthy man. Hb. 96, RBC 5.2 million, C. I. 0.93.

Cell types	% non-labeled cells	% labeled cells 1+	2+	3+	4+	% total labeled cells	Relative activity (total +'s)
						After 2 hr incubation	
Proerythroblasts	57	31	11	1	0	43	56
Basophilic erythroblasts	42	45	12	1	0	58	72
Polychromatic	40	53	7	0	0	60	67
Orthochromatic	55	38	7	0	0	45	52
						After 4 hr incubation	
Proerythroblasts	52	38	8	2	0	48	60
Basophilic erythroblasts	39	35	15	10	1	61	99
Polychromatic	38	41	14	7	0	62	100
Orthochromatic	28	41	18	12	1	72	117
						After 9 hr incubation	
Proerythroblasts	48	45	6	1	0	52	60
Basophilic erythroblasts	47	43	6	4	0	53	67
Polychromatic	37	51	11	1	0	63	76
Orthochromatic	29	54	13	3	1	71	93

The 12 and 24 hr incubation samples yielded defective preparations and were discarded.

cedure(1). After 15-30 days, the autoradiographs were read by a semi-quantitative estimation method based on the qualitative evaluation of *non-labeled* (or negative) and labeled cells, and on the 1^+, 2^+, 3^+, 4^+ quantitative classification of the marked cells according to their relative autoradiographic activity. The sum of the plusses of the labeled elements of each erythroblastic stage reflects the cell radioactivity and the differential capacity of each erythroid element to take up iron *in vitro*. Determinations of *labeled haemoglobin* resulting from the above incubation experiments were conducted in a few cases, in order to measure the erythron capacity of synthesizing heme *in vitro*. The same incubation experiments were also performed with the circulating blood of a few subjects in order to ascertain if young erythrocytes are capable of synthesizing heme *in vitro*. For this purpose, after incubation and washing, haemoglobin was liberated from the cellular stroma with distilled water. The haemoglobin solutions were then tested for their radioactivity with a Geiger-Muller or scintillation counter. Direct autoradiographs were also taken of known amounts of dried haemoglobin. When a more purified Hb. was desired, electrophoretic separation at varying pH levels was also performed and the activity of the different fractions determined by counting or autoradiography. With the above technic the marrows and bloods of 15 cases, normal individuals or patients with various abnormal conditions, were examined.

Results. 1. *Cytoautoradiography.* Table I gives data of a typical experiment. As has been demonstrated by *in vivo* studies in the rat(1), the human normal erythroblast *in vitro* also shows the capacity of rapidly taking up considerable amounts of radioiron. All the erythroblastic maturation stages are more or less "labeled" (Fig. 1). The percentage of marked elements varies between 40 and 70%, according to the maturation stage, incubation time, specific activity of the isotope, exposure time and other non-identifiable factors. In other experiments all the elements are similarly labeled, allowing the conclusion that, potentially, all the immature elements of the erythron keep their capacity of utilizing iron *in vitro*. Also a certain number of medullary young erythrocytes with morphological aspect of reticulocytes, show a definite iron-uptake (Fig. 1). Only circulating blood with considerable reticulocytosis showed,

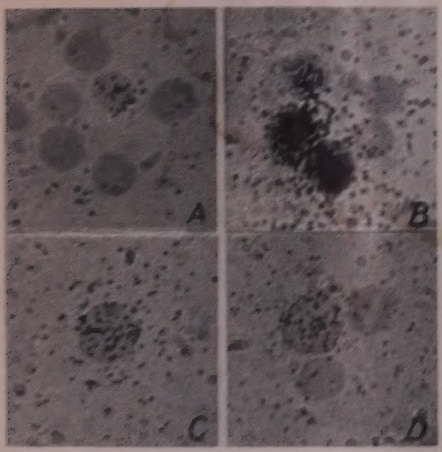

FIG. 1. Bone marrow "labeled" young erythrocytes: autoradiographic picture of the *in vitro* Fe^{59}-uptake by erythrocytes of a normal young woman after 2 hours incubation with 5 μc of Ferrous^{59} citrate. A) Small labeled erythroeyte; B) 2 labeled erythroblasts and a small labeled erythroeyte; C) and D) 2 large polychromatophilic labeled erythroeytes. (Stained autoradiographs; 1000 ×.)

after incubation with radioiron, significant autoradiographic evidence of erythrocyte labeling. A case of Marchiafava-Micheli disease with 96% reticulocytosis revealed considerable labeling. The maximum iron-uptake occurs, after 2 hours incubation, in the basophilic and polychromatic stages (58% and 60% with 72 and 67 plusses, respectively). But, after 4 hours, the orthochromatic elements show a much higher activity (72% with 117 plusses), while the more immature elements undergo a slighter increase. After 9 hours incubation, there is a slight decrease in all the stages, the maximum still occurring in the orthochromatic cells. The individual cell Fe uptake shows some quantitative variations. Sometimes it appears greater in the earlier phases, sometimes in the later stages of maturation. It may be suspected that this can be related to variations in technic (Fe^{59} specific activity and oxidation state, plasma iron binding capacity, incubation time, film exposure and developing, buffering procedure, washing, etc.). Definite conclusions can not be drawn, especially because it was not possible to evaluate many physiological factors which may be involved in the process. These variations are, in fact, much greater in pathological conditions.

Mature erythrocytes in bone marrow as well as in circulating blood are not able to take up radioiron as shown by the negative autoradiographs of such elements. This confirms the work of Hahn(2) and Gowaerts(3). In regard to the *non-erythroid marrow cells* it appears that, in general, there is no evidence of iron-uptake *in vitro*. Only *eosinophilic elements* are occasionally labeled (Fig. 2). This behavior has been observed only in a few cases, namely liver cirrhosis and chronic hepatitis. It cannot be stated that this observation is related to cellular iron turnover. The negative autoradiographs of the other myeloid cells indicate that heme enzymes and other iron compounds have a relatively low iron metabolism in these cells and that the iron quantities involved are below the sensitivity of this autoradiographic method.

2. *Labeled haemoglobin determinations.* a) *Bone marrow haemoglobin* from normal individuals shows, after 2 hours incubation, a specific activity of 0.5-1%. Hyperplastic marrows, as in cases of haemolytic anaemias, yield up to 3-4 times as much labeled Hb. Hypoplastic marrows, as in cases of aplastic or aregenerative anaemias, on the contrary, often show a marked reduction of Hb. synthesis. The Hb. production *in vitro*, as *in vivo*, seems to correspond with the functional state of the marrow.

b) *Peripheral blood haemoglobin* from normal individuals and cases of aregenerative anaemias is produced in very low quantities or not detectable at all. In patients with hyperplastic marrow, on the contrary, distinct amounts of labeled pigment (1-2%) are produced by incubating their blood. This seems to be related to the reticulocytosis present in these cases. Autoradiographs and paper electrophoresis determinations with scintillation countings gave results consistent with the above-mentioned data.

These results demonstrate that at least some part of the iron taken up by the bone marrow or blood reticulocytes is for haemoglobin formation. The work of Watson(4)

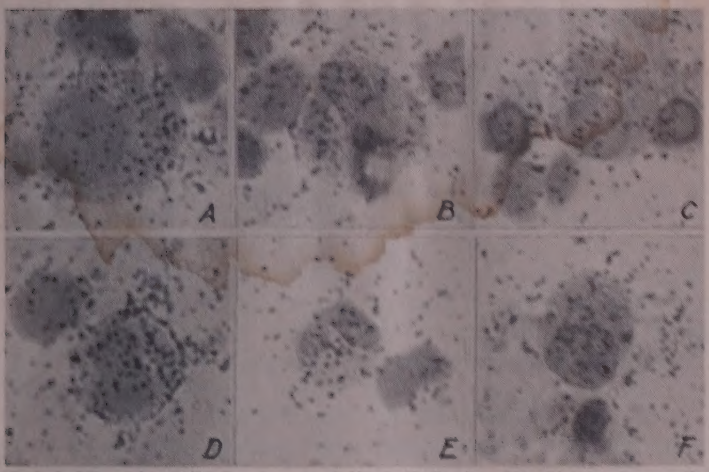

FIG. 2. "Labeled" eosinophils in a case of liver cirrhosis with slight macrocytic anaemia: A, B, C—earlier and D, E, F—later medullary eosinophils after 2 hours incubation with 5 μc of Ferrous^{59} citrate. (Stained autoradiographs; × 1000.)

has pointed out that in the erythron there are, as precursors of heme, protoporphyrin, globin and Fe. As long as these compounds are present the synthesis takes place. The anuclear mammalian erythrocyte is usually released into the circulation at a time which approximates the completion of haemoglobin formation. Presumably, no further Hb. formation occurs after that time. But, if for some reason, the erythrocyte is released when still immature, as a reticulocyte or erythroblast, further Hb. synthesis may take place in the circulating blood(5), provided that these red cells contain the other precursor materials. This happens also *in vitro*, as shown by the above experiments. The nucleus then is not necessary for this final step of Hb. synthesis, but it must be pointed out that the nucleus plays the directing role in synthesizing the porphyrin precursors.

Summary. The *in vitro* uptake of Fe^{59} by normal human erythrons is described. All the erythroblastic maturation stages and young erythrocytes in bone marrow as well as in circulating blood take up considerable amounts of radioiron. During the earlier phases of the experiment, the iron-uptake is maximal in the late basophilic and early polychromatic elements. Subsequently, the maximum is reached in the orthochromatic stage. Marrow reticulocytes and circulating young erythrocytes take up small quantities of isotope. Mature erythrocytes do not seem capable of so doing. Iron utilization for haemoglobin synthesis appears to be the main function of the erythron.

1. Austoni, M. E., PROC. SOC. EXP. BIOL. AND MED., 1954, v85, 48; *Wien. Z. Inn. Med.*, 1954, v35, 151. Austoni, M. E., e Ziliotto, D., *Acta Med. Patavina*, 1955, in press.

2. Hahn, P. F., Bale, W. F., Ross, J. F., Hettig, R. A., and Whipple, G. H., *Science*, 1940, v92, 131.

3. Gowaerts, J., et Lambrechts, A., *Acta Biol. Belgica*, 1943, v3-4, 209.

4. Watson, C. J., *Arch. Int. Med.*, 1950, v86, 797.

5. London, I. M., Shemin, D., and Rittenberg, D., *J. Biol. Chem.*, 1950, v183, 749.

Received November 17, 1954. P.S.E.B.M., 1956, v92.

Distribution of Mucinolytic Activity in Strains of Shigella. (22368)

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That strains of *Vibrio comma* elaborate mucinolytic enzymes was first noted by Burnet and Stone(1). This finding has since been confirmed and extended by a number of investigators so that it is now established that: a) the mucinolytic enzyme is able to produce a desquamation of the intestinal mucosa of guinea pigs(1) and increase the permeability of the intestinal wall of mice(2); b) intestinal contents of animals experimentally infected with cholera organisms possess demonstrable mucinolytic activity(3); c) sera of patients convalescing from an attack of cholera contain antibodies which neutralize the action of this enzyme(4) and d) that at least two serological types of mucinase are produced by *V. comma*(5). The possibility is therefore recognized that mucinolytic enzymes may be implicated in the pathogenesis of Asiatic cholera.

The characteristics of bacillary dysentery are similar in certain respects to those of cholera. Whether this similarity includes the elaboration of mucinolytic enzymes by members of the genus *Shigella* is the subject of this communication.

Material and methods. Cultures. The cultures employed were obtained from 3 sources: the culture collection of the Walter Reed Army Institute of Research, the 406th Medical General Laboratory in Tokyo, and The Hospital Infantil, in Mexico City.* The strains obtained in the Hospital Infantil were tested for mucinolytic activity immediately after isolation. *Ovomucin.* Ovomucin, as a substrate for the mucinolytic enzymes, was prepared by the method described elsewhere (5,6). *Mucinase.* Cultures to be tested for mucinolytic activity were grown on brain heart infusion soft agar (0.7% agar). After 18 hours incubation at 37°C the liquid was expressed from the soft agar medium and cleared by centrifugation. The supernatant was tested for mucinolytic activity. The mucinase obtained from one culture was concentrated for use in immunization of rabbits. A 10-fold concentration was accomplished by adding cold acetone (60% by volume) to the chilled supernatant and dissolving the resulting precipitate in distilled water at one-tenth the original volume. *Mucinase titrations.* The fact that intact ovomucin but not depolymerized ovomucin is precipitated or clotted by cetyl trimethyl ammonium bromide (CTAB) served as a basis for the mucinase titrations(7). One half ml of ovomucin was added to 0.5 ml of 2-fold serial dilutions (in borate-buffered saline, pH 7.0)† of culture supernatants. After one half hour incubation in a water bath at 40°C, undigested ovomucin was detected by the addition of 1 drop of 0.1% aqueous solution of CTAB. The highest dilution of supernatant in which the addition of CTAB failed to precipitate or clot the ovomucin was recorded as the mucinase titer.

Antimucinase titration. Culture supernatants were diluted in buffered saline to yield a solution with a mucinase titer of 1:8 (8 units per ml). Aliquots of 0.25 ml (2 units) of such preparations were added to 0.25 ml of two-fold serial dilutions of the antiserum. Following incubation at room temperature for 1½ hours, 0.5 ml ovomucin was added to each tube and the test incubated at 40°C for one half hour. The test was read in the manner here described except that the titer of the antiserum was taken as the highest dilution in which a clot was observed, indicating neutralization of the mucinolytic enzyme.

Results. Mucinolytic activity by strains

* The authors wish to express their thanks to the Commanding Officer of the 406th Medical General Laboratory and to Dr. F. Gomez and Prof. J. Olarte of the Hospital Infantil, for making available laboratory facilities for the field aspects of this study.

† .052g $Na_2B_4O_7 \cdot 10H_2O$
1.203g H_3BO_3
1.0 g $CaCl_2$
8.5 g NaCl
1000 ml Distilled H_2O

TABLE I. Distribution of Mucinolytic Activity in Strains of *Shigella*.

Culture type		Source* Stock collection No. pos.	Stock collection No. neg.†	Tokyo No. pos.	Tokyo No. neg.	Mexico City No. pos.	Mexico City No. neg.	Total tested	Total pos.
Sh. dysenteriae	1	0	7					7	0
	2	0	14					14	0
	3	0	6					6	0
	4	0	7					7	0
	5	0	2					2	0
	6	0	6					6	0
	7	0	5					5	0
Sh. flexneri	1a	0	7	0	1			8	0
	1b	0	15					15	0
	2a	8	3	8	0	8	0	27	24
	2b	0	7	0	5	0	2	14	0
	3	0	13	0	7	0	2	22	0
	4	0	20	0	1	0	1	22	0
	5	0	3			0	1	4	0
	6	0	17					17	0
	X	1	3					4	1
	Y	2	7					9	2
Sh. boydii	1	0	2					2	0
	2	0	2					2	0
	3	0	2					2	0
	4	0	5					5	0
	5	0	6					6	0
	6	0	4					4	0
	7	0	8					8	0
Sh. sonnei	I	0	29	0	6	0	1	36	0

* Cultures from stock culture collection were tested for mucinolytic activity after being maintained on laboratory media for years; cultures obtained in Tokyo were tested approximately 10 months after isolation; strains from Mexico City were tested for mucinolytic activity immediately after isolation.

† Strains with mucinolytic titers which were less than 1:8 were recorded as negative, since on occasion 1:4 dilutions of supernatants of uninoculated medium inhibited the precipitation of ovomucin by CTAB. Thus, it is possible that many strains of *Shigella* produce mucinolytic enzymes in concentrations too small to detect by the method employed.

of *Shigella* was first observed in a culture of *Sh. flexneri* 2a.‡ As a result of this finding a survey of available strains was carried out. Table I gives the results of this study. It will be noted that, under the conditions of growth which were employed, most of the strains of *Sh. flexneri* 2a elaborated the enzyme. A few strains of *Sh. flexneri* X and Y possessed activity, whereas no other strains manifested detectable mucinase. The mucinolytic titer of the crude Shigella supernatants varied from 1:8 to 1:32 thus exhibiting less activity than similarly prepared cholera supernatants.

An immune serum was prepared in rabbits by immunization with a concentrate of the crude supernatant of *Sh. flexneri* 2a, strain 2-2. This antiserum (titer = 0.25 ml of a 1:320 dilution) was capable of neutralizing the mucinolytic activity of 2 units (contained in 0.25 ml) of the homologous mucinase. Serum taken before immunization was inactive in a dilution of 1:20, and antimucinase activity was not observed with an immune serum (agglutinin titer 1:1280) prepared against living cells of strain 2-2. Furthermore, adsorption of the antimucinase serum with viable 2-2 cells caused no reduction in its antimucinase titer indicating that mucinase is antigenically distinct from the somatic antigen. An investigation is now in progress to determine whether this antimucinase serum will neutralize the mucinases produced by other strains of *Sh. flexneri*.

Discussion. Relatively little is known of

‡ While this study was in progress Freter(8) also observed mucinolytic activity in a culture of *Sh. flexneri* 2a.

the pathogenesis of bacillary dysentery. At one time considerable importance was placed on the extreme toxicity of the endotoxin of dysentery bacilli. However, this now seems unwarranted, inasmuch as it has been shown that the somatic antigens of all enteric organisms, nonpathogens as well as pathogens are more or less of the same order of toxicity (9). Thus, while it would be incorrect to assume that the somatic antigen plays no part in pathogenesis, one must look beyond it to determine why organisms of the Shigella group cause disease while most strains of *Escherichia coli*, for instance, do not.

The observation that a strain of *Sh. flexneri* 2a possesses mucinolytic activity suggested that this enzyme may play some part in pathogenesis. This has provided the impetus for the study here reported. Under the conditions of growth described, however, only certain strains of *Sh. flexneri* 2a, X and Y produced the enzyme while all available cultures of the closely related *Sh. flexneri* 2b failed to do so. The length of time the culture had been maintained on laboratory media made little difference, for both stock and freshly isolated strains of *Sh. flexneri* 2a possessed mucinolytic activity while stock and freshly isolated cultures of *Sh. flexneri* 2b, 3, 4, 5, and *Sh. sonnei* were negative.

On the basis of these preliminary studies, therefore, it is not possible to assess the role of mucinase in the pathogenesis of bacillary dysentery. If this particular enzyme is of any great importance in participating in the establishment of infections by the organism, it seems likely that it would be elaborated by most if not all serological types, rather than by only 2 or 3. It is of course possible that the capacity to produce mucinase is rapidly lost by most strains on artificial cultivation, or that types of dysentery bacilli other than *Sh. flexneri* 2a, X and Y will produce mucinolytic enzymes only under conditions of growth different from those employed in this study. Such possibilities remain to be investigated.

Summary. A survey was made to determine the incidence of mucinase production among representative strains of the genus Shigella. With the culture conditions employed, 24 out of 27 strains of *Sh. flexneri* 2a, 1 of 4 cultures of *Sh. flexneri* X and 2 of 9 strains of *Sh. flexneri* Y demonstrated mucinolytic activity while none of the 214 strains of other serological types of dysentery bacilli were found to be active. Immunization of rabbits with a crude enzyme preparation from a strain of *Sh. flexneri* 2a yielded an antiserum which neutralized its mucinolytic activity.

1. Burnet, F. M., and Stone, J. D., *Austral. J. Exp. Biol. and Med. Sci.*, 1947, v25, 219.
2. Lam, G. T., Mandle, R. J., and Goodner, K., *J. Inf. Dis.*, 1955, v97, 268.
3. Freter, R., *ibid.*, 1955, v97, 57.
4. Lowenthal, J. P., unpublished data.
5. Freter, R., *J. Inf. Dis.*, 1955, v97, 238.
6. Lowenthal, J. P., in mss.
7. Burnet, F. M., *Austral. J. Exp. Biol. and Med. Sci.*, 1949, v27, 245.
8. Freter, R., personal communication.
9. Burrows, W., *Ann. Rev. Microbiol.*, 1951, v5, 197.

Received April 3, 1956. P.S.E.B.M., 1956, v92.

Radiosensitivity of Larval and Adult Amphibia in Relation to Temperature During and Subsequent to Irradiation.* (22369)

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That the temperature prevailing in a protoplasmic system *subsequent* to exposure to ionizing radiations is an important factor in modifying the extent of damage reflected over a given period is generally acknowledged. However, regarding the effect of temperature *during* exposure, Patt has written recently and fittingly: "Evidence that relates to the effect of temperature during exposure to radiation is equivocal and virtually every conceivable effect has been described"(1). The issue is highly important toward an understanding of the nature of biological responses to ionizing radiation. Patt and Swift(2) have presented good evidence that (a) survival of frogs (*R. pipiens*) is not influenced by altering their body temperature during and/or for the first 24 hours following exposure to X-rays, and (b) survival is greatly enhanced so long as the animals are kept at 5°-6°C, continuously, following exposure. When, after 60 to 130 days in the cold these latter are returned to 23°C, there is no change in absolute survival nor any significant difference in the time-course of deaths when compared with similarly irradiated frogs which had been maintained at 23°C immediately following irradiation. It would be of interest to learn whether this pattern of post-irradiation response would be duplicated by larval frogs. In preliminary communications (3-5) we have reported that reduced temperature during exposure to X-rays was accompanied by increased radioresistance in urodele larvae (Amblystoma); this was reflected in such criteria as feeding behavior, response to tactile stimuli, swimming motions, atrophy of gills and tail, and damage to epithelia of digestive tract and tissues of the eye and brain. While no mortality studies were made, the observations are rather at variance with what might reasonably be expected on the basis of Patt and Swift's data on adult frogs. Also, the histopathologic data on these urodele larvae do not agree with the findings of Allen and his associates(6) on the radiosensitivity of hematopoietic tissue of tadpoles of *R. catesbiana* irradiated at different temperatures.

Accordingly, the present report will be concerned with: (a) influence of temperature prevailing during and after whole-body irradiation on X-ray lethality in anuran larvae; (b) influence of temperature during and after whole-body X-irradiation as reflected in certain tissues and features of behavior of larval urodeles and (c) survival characteristics of adult urodeles irradiated at different temperatures.

Materials and methods. Larvae of *R. catesbiana* (predominantly first year), larval *A. punctatum* and *A. maculatum* (15-22 mm) and *Triturus viridescens* comprised the experimental material. Temperature of the surrounding water was taken as the measure of body-temperature during and subsequent to irradiation. Animals to be irradiated at 2°-3°C or 7°-9°C were conditioned to these temperatures for at least an hour before exposure. Irradiation conditions and disposition of control groups will be more conveniently described below in connection with each experiment.

Results. Exp. 1A. *Larval R. catesbiana exposed to 1000r at 3°C, and 23°C, respectively, and subsequently maintained at ca 25°C.* Irradiation conditions; 120 kv; 9 ma; target-specimen distance, 25 cm (measured to half the 9 cm depth of water in a plastic container, 15 cm in diameter); no added filter; 200r/min.; dose, 1000r. The results, and control data are summarized in Fig. 1, It will be seen that the temperature at the

* This investigation was supported by the James Picker Foundation on recommendation of Committee on Radiology, National Research Council.

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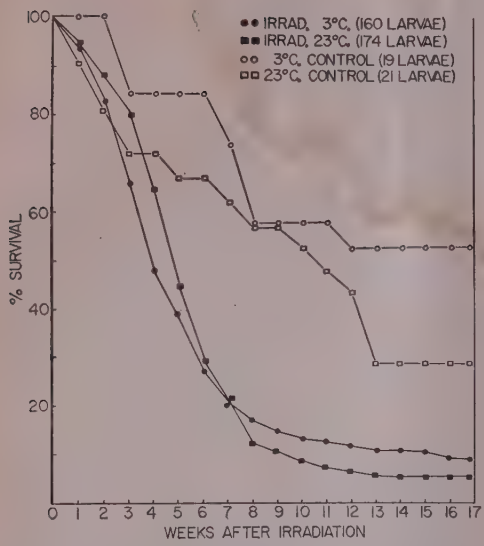

FIG. 1. Survival characteristics of larval *R. catesbiana* irradiated at 3°C and 23°C, respectively, and subsequently maintained, at 25°C; non-irradiated control groups also shown.

time of exposure had no significant effect upon the survival characteristics of larvae maintained at ca 25°C subsequent to exposure.

Exp. 1B. *Larval R. catesbiana exposed to 1000r at 3°C and 23°C, respectively, and subsequently maintained at 3°C for 8 weeks and then returned to ca 25°C.* Irradiation conditions were the same as for Exp. 1A. The results, and control data, are summarized in Fig. 2. Clearly, temperature at the time of exposure had no significant effect on survival when the animals were kept, subsequent to exposure, at 3°C for 8 weeks and then returned to room temperature; prolonged refrigeration after irradiation unmistakably fostered survival, *but only for such time as the reduced temperature prevailed.* Each control group here, and in Exp. 1A exhibited only ca 50% survival at 11-12 weeks; this will be discussed below.

Exp. 2A. *Larval A. punctatum exposed to 1176r or 3136r at 7°-9°C and 26°-28°C, respectively; after irradiation half of each group was maintained at 12°-15°C and half at 22°-24°C; appropriate control groups were observed.* Irradiation conditions: 140 kv; 5 ma; 1000r/min. Larvae were anesthetized in 1:2000 MS 222 during exposure. No survival studies were made. Observations based on 360 specimens, of which some 50 were periodically fixed in Bouin's and studied histologically. Observations on living larvae: in virtually all of the specimens studied, the following patterns of externally observable responses were clearly evident. As compared with larvae irradiated at the lower temperature those irradiated at the higher temperature exhibited: (a) markedly reduced readiness (ability?) to feed on small enchytraeid worms which are ordinarily a favorite food of such larvae; (b) decidedly more abnormal swimming movements, whether spontaneously inaugurated or in response to tactile stimuli; (c) a more pronounced curling and darkening of the extremity of the tail and also a more marked atrophy of the gills. These manifestations were not found in control groups. Histological observations: Particular attention was directed to the epithelial elements of the digestive tract, for these are generally regarded as particularly radiosensitive and their relative simplicity enhances their value in the assaying of cellular damage. Here too the damage is noticeably greater in

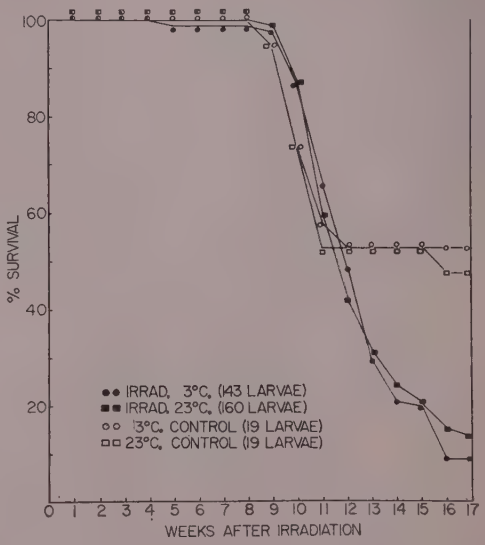

FIG. 2. Survival characteristics of larval *R. catesbiana* irradiated at 3°C and 23°C, respectively, and subsequently maintained at 3°C for 8 weeks and then returned to 25°C; non-irradiated control groups also shown.

those specimens irradiated at the higher temperature; damage is reflected principally in a variety of chromatin disarrangements, vacuolization of the cytoplasm and overall tissue degeneration.

Exp. 2B. *Larval A. maculatum exposed to 15,750r at 2°C, and 33°C, respectively, and subsequently maintained at ca 25°C; appropriate non-irradiated controls were observed.* Irradiation conditions: 140 kv; 5 ma; target-specimen distance 11.5 cm; 1050r/min. (This experiment is essentially the same as Exp. 2A except for a much heavier dose of radiation and a wider range between the two temperatures employed.) The larvae exposed at 33°C exhibited such abnormal features as curling and darkening of the extremity of the tail and abnormal swimming movements as early as 1 day following irradiation, whereas these changes appeared in the group exposed at 2°C only 3 days following exposure. A histological study was directed to the tissues of the eye and the wall of the forebrain; the damage to the larval eye and brain wall was significantly less, over a given time, in those larvae exposed at the lower temperature. The damage was most clearly revealed in the developing retinal layers of the eye.

Exp. 3. *Adult T. viridescens exposed at 3°C and 26°C, respectively, and subsequently maintained at ca 25°C.* Irradiation conditions: 220 kv; 15 ma; target-specimen distance, 25 cm (measured to half the 9 cm depth of water in a circular plastic container, 15 cm in diameter); 396r/min.; dose, 7920r. The experimental results, with control data, are summarized in Fig. 3. It will be seen that the temperature at the time of irradiation had a definite effect on the time-course of deaths; the lower exposure-temperature was accompanied by a significantly lower mortality rate during the first 5 post-irradiation weeks.

Discussion. The results of Exp. 1A demonstrate that, for the temperature points and radiation dosage employed, the temperature at which larval *R. catesbiana* were irradiated was without significant influence on the final mortality outcome. In Exp. 1B, prolonged refrigeration following irradiation was accompanied by the same enhancement of survival (during the period of refrigeration) as was described for adult frogs by Patt and Swift(2). The present results with anuran larvae are in full agreement with the observations of these authors and, we believe, are of additional value by reason of the larger number of organisms employed. The data would suggest that the primary changes, effected during the irradiation, are independent of body temperature and that the enhanced survival during prolonged refrigeration following exposure reflects a postponement or retardation of temperature-sensitive secondary reactions, rather than any real measure of "recovery."

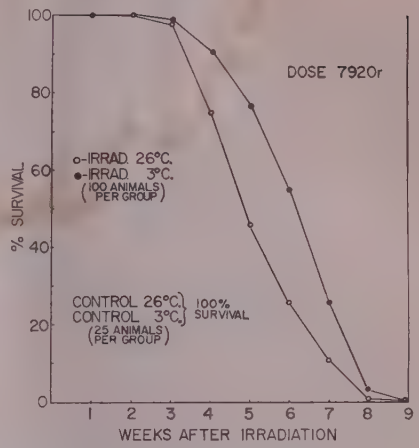

FIG. 3. Survival characteristics of adult *T. viridescens* irradiated at 3°C and 26°C, respectively, and subsequently maintained at 25°C; there was 100% survival in the non-irradiated control groups.

It will be noted that in Exp. 1A the non-irradiated control groups exhibited only about 55% survival at 11-12 weeks. While this reflects a rather high mortality, it is definitely considerably less than the approximate 90% mortality exhibited over this same period by the experimental groups; these latter showed a 50% mortality as early as 4-5 weeks following irradiation. It may also be pointed out that at about the 13th week the control and experimental groups level off and continue with relatively little change for another month at which time the experiment was terminated. While this mortality rate in the controls was higher than might be an-

ticipated, it would seem that the overall time course of deaths in the controls together with the absolute survival figure to 17 weeks are sufficiently distinctive so as to suggest that the factors involved did not essentially modify the survival characteristics of the irradiated larvae. It is important to note that in Exp. 1B the control and irradiated larvae both exhibited 50% mortality at 11-12 weeks. However, at this point the controls leveled off and maintained this 50% survival picture to 17 weeks. whereas the irradiated larvae rapidly declined to an approximate 10% survival by week 17. It would appear that under the existing laboratory conditions, 50% survival at 11-12 weeks and a leveling off from that point onward is a characteristic response of non-irradiated bullfrog larvae and need not be regarded as affecting the survival data of the irradiated animals.

The results of Exp. 2A and 2B (it will be remembered that no survival studies were made on these groups) are not easily reconciled with the present data on frog larvae, and the studies of Allen, *et al.*(6) on radiosensitivity of hematopoietic tissues in the tadpole under different conditions of temperature. They do agree, however, with data gathered by others in similar studies involving embryonic chick tissue, amphibian germs and eggs of drosophila.

The results of Exp. 3 with adult *T. viridescens* present a different picture. Here it will be seen that when the organisms are irradiated at 3°C the mortality-rate is significantly lower during the first 5 post-exposure weeks than in those animals irradiated at 26°C, both groups having been returned to room temperature following exposure.‡ More specifically, at the end of the 5th post-irradiation week, the mortality is about 50% for those animals exposed at 26°C, whereas it is only about 20% for those exposed at 3°C. These results are in agreement with the earlier noted observations on larval urodeles in Exp. 2A and 2B. They do not agree with the data of Patt and Swift on adult frogs(2). While the temperature at the time of exposure did not, in terms of absolute survival, affect the final outcome, nevertheless, the significant difference in the time-course of deaths over a representative post-irradiation period (5 weeks) is evidence that the temperature prevailing *during* irradiation is a radiosensitivity-modifying factor.

We are not prepared at this time to suggest an explanation for the difference in response, following irradiation at different temperatures, of adult and larval anurans on the one hand and larval and adult urodeles on the other.

Summary. 1. Survival of larval *R. catesbiana* is not affected by regulation of body temperature (to 3°C or 23°C) during whole-body irradiation; prolonged refrigeration subsequent to irradiation fosters survival, but only for as long as the animals are kept at the reduced temperature (3°C); upon return to 23°C the mortality characteristics develop in essentially the same pattern as larvae maintained at 23°C immediately subsequent to irradiation. 2. As compared with those irradiated at lower temperatures, 2-9°C, larval *A. punctatum* and/or *A. maculatum* irradiated at higher temperatures, 28-33°C, and subsequently maintained at room temperature, exhibit definitely more abnormal (externally observable) responses, as well as greater damage to digestive epithelium and elements of the developing eye and brain wall. 3. Adult *T. viridescens* irradiated at 3°C exhibit, during the first 5 post-irradiation weeks, a significantly lower mortality rate than those irradiated at 26°C, both having been returned to 23°C immediately after exposure.

‡ We are indebted to Dr. Joseph Talacko, Department of Mathematics, Marquette University, for his assistance in the statistical analysis of these data.

1. Patt, H. M., in *Radiation Biology* (A. Hollaender, Editor), 1955, v1, 19.

2. Patt, H. M., and Swift, M., *Am. J. Physiol.*, 1948, v155, 388.

3. O'Brien, J. P., *Anat. Rec.*, 1947, v99, 651.

4. ———, *ibid.*, 1949, v105, 553.

5. O'Brien, J. P., and LaHam, Q., *ibid.*, 1951, v111, 496.

6. Allen, B. M., Schjeide, O. A., and Hochwald, L. B., *J. Cellular Comp. Physiol.*, 1951, v38, 69.

Received November 2, 1955. P.S.E.B.M., 1956, v92.

Difference Between Dorsal and Lateral Components of Dorsolateral Prostrate of Rat in Zn^{65} Uptake.*† (22370)

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In a previous communication it was reported that the dorsolateral prostate of the rat concentrates Zn^{65} from 15 to 25 times more than any other tissue(1). In view of the observation that there is a marked difference grossly and microscopically between the dorsal and lateral portions of the dorsolateral prostate, the possibility of a functional difference in the capacity of the two glandular components to take up Zn^{65} was investigated.

Procedure. Eighteen male Wistar rats (350-450 g) were used in these studies. The technic of Zn^{65} experiments was similar to that outlined previously(1,3). Zn^{65} (0.4 μc/g)‡ was administered by intracardiac injection. The rats were sacrificed by chloroform and exsanguination at various intervals post-injection, 20 min, 40 min, 2, 4, and 24 hrs. The right and left dorsolateral lobes of the prostate were exposed and on one side the intact dorsolateral lobe was removed for study. On the other side the dorsal and lateral portions were separated for individual study. With experience it became easy to differentiate the blanched area dorsal to the urethra from the pink glistening lateral portion of the lobe. The dissected tissues were placed in metal planchets, dried overnight at 110°C, and weighed. Radioactivity was measured by an end-window Geiger-Müller counting tube in conjunction with a decade scaler. The experimental results are expressed in the Figures as counts/min/mg of tissue. For the histological study of the dorsolateral prostate, tissues were fixed in 10% formalin and stained by routine hematoxylin and eosin methods. The histochemical study for zinc was carried out according to the technics outlined by Mager, McNary and Lionetti (4).

Results. The results show (Fig. 1) a striking difference in the degree of uptake of radioactive zinc by the lateral and dorsal portions of the dorsolateral prostate 24 hrs after the intracardiac administration of Zn^{65}. The dorsal portion of the dorsolateral prostate is satu-

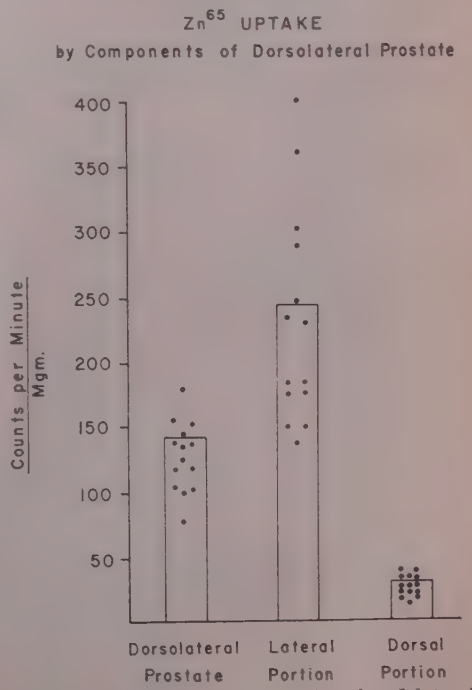

FIG. 1. 24-hr uptake of Zn^{65} by dorsal and lateral components of dorsolateral prostate of mature rat.

* This investigation was supported by U. S. Atomic Energy Commission, the Committee on Research, Council on Pharmacy and Chemistry, American Medical Assn., and the Schering Corp.

† The authors wish to express their appreciation to Dr. Alfred P. Mills, Director of the Radioisotope Laboratory, for his cooperation, and to Miss Maureen Sweeney, R. T. (Can.) Department of Pathology, for her valuable technical assistance.

‡ Zn^{65} was purchased from Carbide and Carbon Chemicals Co. as $Zn^{65}Cl_2$ in HCl solution with a specific activity of 236 mc/g. The solution was diluted with 0.85% NaCl containing sufficient NaOH to neutralize some of the acid present compatible with solubility. The dilution was made so that the administered dose was contained in less than 0.4 ml.

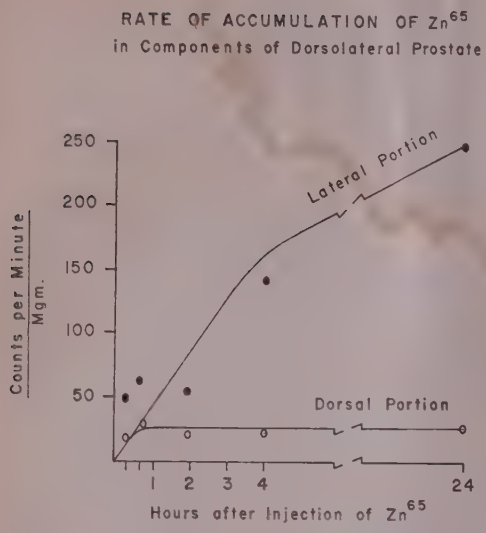

FIG. 2. Comparison of rate of accumulation of Zn^{65} in dorsal and lateral components of dorsolateral prostate of mature rat.

rated with a very low level of Zn^{65} by 20 to 40 min after injection of the radioactive material (Fig. 2), whereas the lateral portion of the gland continues to accumulate Zn^{65} until 4 to 24 hrs post-injection. Results of an earlier study(1) showed that the concentration of Zn^{65} in the whole dorsolateral prostate does not increase after 24 hrs.

In view of this striking difference between the two portions of the dorsolateral prostate to concentrate Zn^{65}, a more intensive histological study of the gland was undertaken. As mentioned previously, in gross dissection the dorsal part of the gland can be differentiated by its lack of pink coloration so evident in the lateral portion. The two parts of the gland also differ histologically. In the lateral portion (Fig. 3a) the cells are tall columnar with large vesicular nuclei, and the lumina are filled with acellular, homogeneous material as seen in apocrine glands. In contrast the cells of the dorsal portion (Fig. 3b) are low cuboidal with more pyknotic nuclei, and the material within the acini is composed of desquamating cells in various stages of disintegration, the typical picture of a holocrine gland.

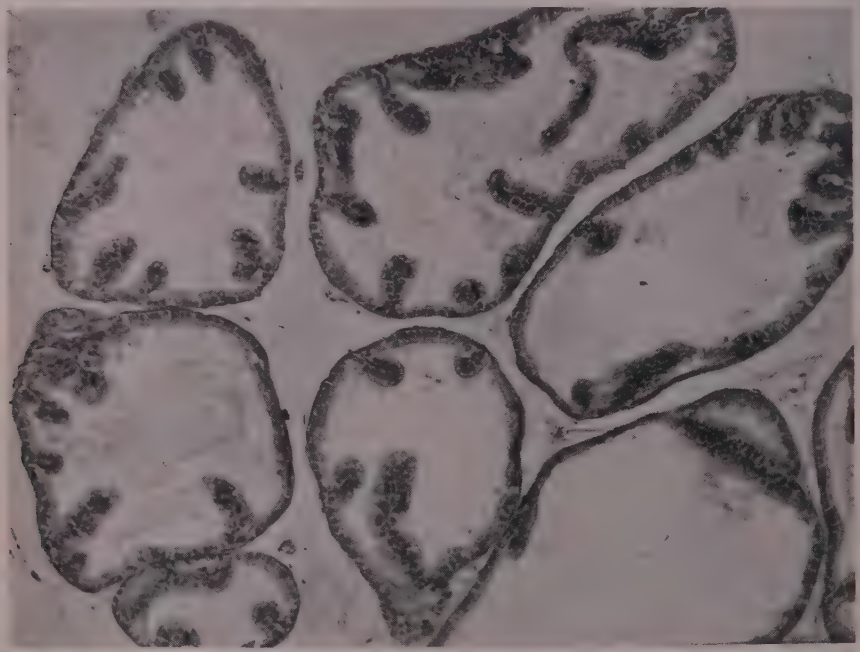

FIG. 3a. Lateral portion of dorsolateral prostate of mature rat showing acini lined by tall columnar cells and lumina filled with acellular, homogeneous secretion. Hematoxylin and eosin × 136.

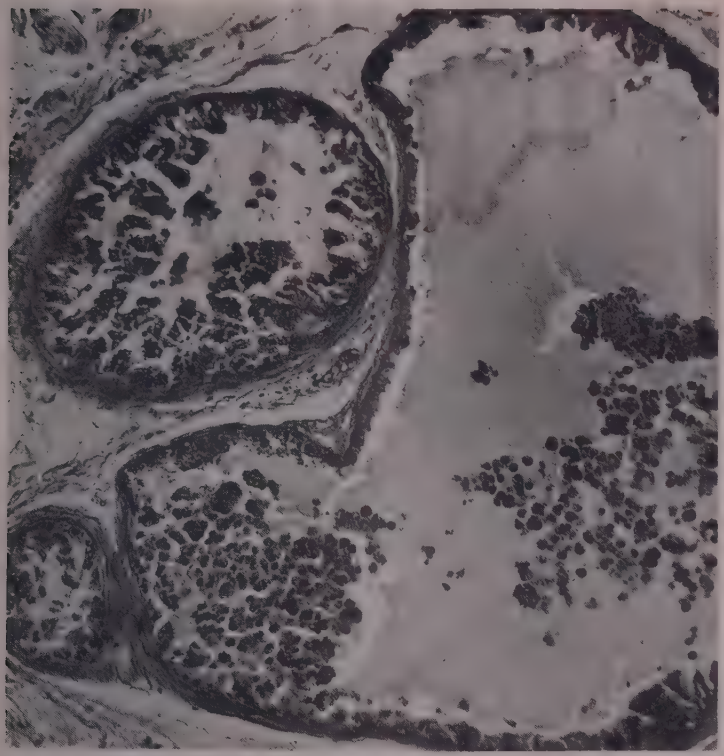

FIG. 3b. Dorsal portion of dorsolateral prostate of mature rat, showing acini lined by low cuboidal cells with pyknotic nuclei and secretion composed of desquamating cells in various stages of disintegration. Hematoxylin and eosin × 136.

Histochemical studies with the dithizone zinc stain show an intense positive reaction in the lateral portion in contrast to a negative reaction in the dorsal portion of the dorsolateral prostate (Fig. 4).

Discussion. Although the dorsolateral prostate is seemingly one lobe enclosed within one capsule, the data reported indicate marked differences between the dorsal and lateral components of the gland. Grossly the lack of pink coloration in the dorsal portion distinguishes it from the adjacent lateral component. Microscopically the cells of the dorsal portion can be distinguished from those of the lateral portion(5). Moreover histological evidence presented in this paper illustrated that the two components of the gland differ strikingly in their manner of secretory formation.

Fischer, Mawson and Tikkala(2) reported that the lateral part of the gland contained more zinc than the dorsal portion, and histochemical studies presented here confirm their observation. In addition the results of studies with radioactive zinc suggest there is a functional difference between the two portions of the dorsolateral prostate in view of the fact that the lateral component of the gland can concentrate administered Zn^{65} to a much greater degree than the adjacent dorsal portion.

Summary. The authors reported previously that the dorsolateral prostate of the rat concentrates administered Zn^{65} from 15-25 times more than any other tissue(1). The present study reveals that this selective uptake of Zn^{65} is confined to the lateral portion of the dorsolateral prostate, suggesting a functional difference between the two parts of the gland. Histological study also lends evidence to this

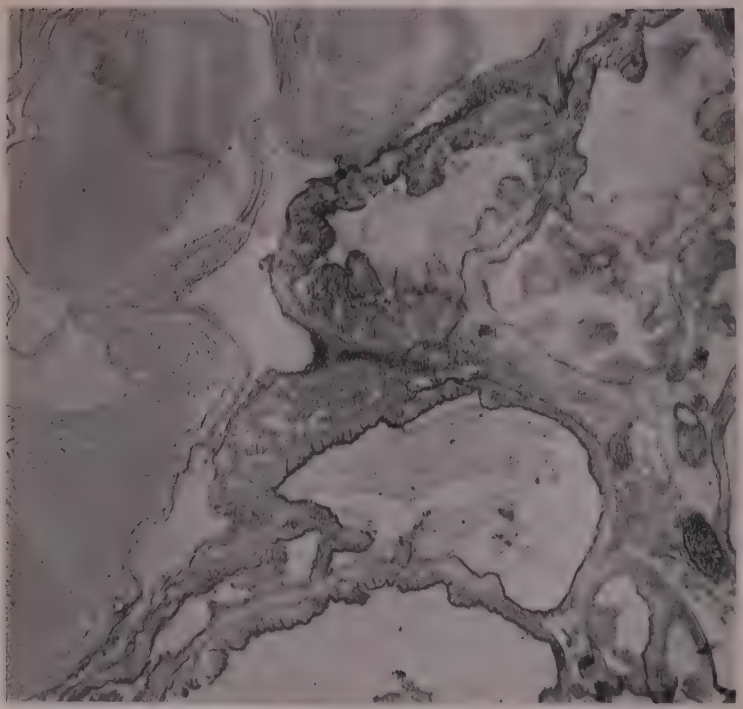

FIG. 4. Zinc stain of dorsolateral prostate of mature rat at junction of dorsal (left) and lateral (right) components of the gland, showing positive zinc stain in the lateral portion (black in photo) and negative reaction in dorsal portion (light gray in photo). Dithizone × 136.

observation.

1. Gunn, S. A., Gould, T. C., Ginori, S. S., and Morse, J. G., PROC. SOC. EXP. BIOL. AND MED., 1955, v88, 556.

2. Fischer, M. I., Tikkala, A. O., and Mawson, C. A., *Canad. J. Biochem. and Physiol.*, 1955, v33, 181.

3. Gunn, S. A., and Gould, T. C., *Endocrinology*, 1956, v58, 443.

4. Mager, M., McNary, W. F., Jr., and Lionetti, F., *J. Histochem. and Cytochem.*, 1953, v1, 493.

5. Korenchevsky, V., and Dennison, M., *J. Path. and Bact.*, 1935, v41, 323.

Received April 3, 1956. P.S.E.B.M., 1956, v92.

Inhibition of Insulin Degradation by Amino Acids and Related Compounds.* (22371)

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Shmidt and Saatchian(1) and Mirsky and Broh-Kahn(2) have shown that insulin is readily inactivated by liver preparations at pH 7.5. Insulin labeled with I^{131} is likewise degraded and the accumulation of radioactive material soluble in 10% trichloracetic acid has been found(3,4) to parallel the biological inactivation of insulin and the formation of non-protein nitrogen from insulin. These ob-

* This research project has been supported in part by grants from the U. S. Public Health Service, the Atomic Energy Commission and the Upjohn Co.

servations suggest that this inactivation of insulin is associated with proteolysis and that liberation of TCA-soluble radioactivity from insulin-I^{131} can be used to measure this process. Although this insulin degradation has been attributed by Mirsky and Broh-Kahn (2) to an enzyme, which they called "insulinase," the enzymatic action is probably not absolutely specific for insulin, since α-corticotropin, casein, glucagon and somatotropin have been found to serve as substrate competitors(5,6). In this manner these compounds decrease the action of the enzyme on insulin. The discovery of a compound that could be given safely, orally, and that would significantly inhibit the insulin-degrading enzyme system might be of great advantage in the treatment of diabetes. Indeed, we have produced evidence in this laboratory to show that this mechanism can account for part of the effectiveness of p-aminobenzenebutylurea (7) and of butyltolylsulfonylurea in the treatment of diabetes. Mirsky(8) has reported on the "insulinase" inhibitory effects of several compounds, including a preparation from the liver, of small molecular weight, which he called "insulinase inhibitor."

In studying a certain type of cheese that a patient claimed benefited her diabetes, we found by *in vitro* studies that insulin-I^{131} degradation was not only inhibited by the cheese but also by some of its bacteria and the culture media used to grow the bacteria. Two of the constituents of the media, trypticase and phytone also exerted significant inhibition. Since trypticase is a tryptic digestion product of casein we investigated a casein hydrolysate preparation (Amigen) consisting chiefly of individual amino acids. This compound was found markedly inhibitory and has also been reported to produce hypoglycemia in normal subjects, following its oral ingestion (9). We then investigated the inhibitory effect of many of the individual amino acids and these results serve as the basis for this report.

Methods. The insulin-degrading enzyme preparation used in all experiments consisted of a dried powder remaining after special treatment of beef liver homogenate with acetone and ether. The portion of 4 mg of this material soluble in 0.5 ml of buffer was placed in a beaker. Either 0.067 M phosphate or, much more frequently, 0.1 M Tris (hydroxymethyl) aminomethane, containing 5×10^{-3} M versene was used as buffer. To the enzyme preparation was added 0.5 ml solution of the test inhibitor and 1 ml of insulin solution containing 0.4 μg of insulin-I^{131} (dialyzed for several hours immediately before using) and 0.1 mg of amorphous insulin. In this system the quantity of enzyme is the limiting factor in the rate of the reaction. The total 2 ml mixture, with the pH adjusted to 7.5, was placed in a Dubnoff Metabolic Shaker at 37.5°C and shaken for exactly 5 minutes. Fifteen seconds before the end of the incubation 1 ml of 2% human plasma in water was added to insure complete precipitation of insulin, and at the end of the incubation period 3 ml of 20% trichloracetic acid was added. After standing for 30 minutes the mixture was centrifuged and the precipitate washed twice with 1 ml of 5% TCA. The 3 supernatant specimens were pooled and the precipitate dissolved in 30% KOH. The radioactivity in each specimen was measured by a well-type gamma counter. Then a calculation was made of the percentage of the total radioactivity found in the supernatant fractions. The following 3 control studies were conducted with each experiment: (a) 1 ml of buffer plus 1 ml of insulin solution, (b) 0.5 ml of buffer plus 0.5 ml of enzyme preparation plus 1 ml of insulin solution, and (c) 0.5 ml of inhibitor plus 0.5 ml of buffer plus 1 ml of insulin solution. All determinations were conducted at least in triplicate.

Results. As shown in Table I most of the amino acids tested did not inhibit the insulin-degrading activity. There was marked inhibition by ergothioneine, 3,4-dihydroxyphenylalanine, cystine, isoleucine, indole-3-acetic acid, and casein hydrolysate (Amigen). The mechanism by which certain amino acids inhibit insulin degradation is not known. However, since there is evidence, recently summarized(10), suggesting that insulin may be degraded by reduction and/or by a proteolytic process the effect of inhibitors on these

TABLE I. Results of Incubation of Insulin-I^{131} plus Insulin-Degrading Enzyme with and without Test Inhibitors.

Inhibitor	Moles/ml $\times 10^{-2}$	Avg % I^{131} in supernatant: No inhibitor*	Inhibitor†	Avg % inhibition
Glutamine	2	6.1 ± .4	6.1 ± .6	NSD
	5	4.5 ± .3	4.6 ± .3	
Glycine	2.5	6.1 ± .4	6.1 ± .1	"
	5	5.0 ± .1	5.5 ± .3	"
β-Alanine	2.5	6.1 ± .4	5.8 ± .4	"
	5	4.5 ± .3	5.1 ± .2	"
d,l-Asparagine	2.5	6.1 ± .4	6.3 ± .4	"
	5	4.5 ± .3	5.1 ± .3	"
l-Arginine	2.5	6.1 ± .4	6.4 ± .4	"
	5	4.5 ± .3	4.0 ± .3	"
d,l-Serine	2.5	6.1 ± .4	6.4 ± .1	"
	5	5.0 ± .1	5.2 ± .1	"
d,l-Threonine	2.5	6.1 ± .4	6.6 ± .1	"
	5	5.0 ± .1	4.5 ± .1	"
d,l-Leucine	2.5	6.1 ± .4	5.5 ± .1	"
l-Methionine	1	6.9 ± .2	7.6 ± .5	"
	5	4.5 ± .3	4.3 ± .1	"
l-Histidine monohydrochloride	1	6.9 ± .2	7.6 ±1	"
	5	4.5 ± .3	4.4 ± .2	"
Hydroxyproline	2.5	4.2 ± .1	4.0 ± .1	"
	5	4.2 ± .1	4.1 ± .2	"
Tyrosine	.13	4.7 ± .2	4.5 ± .1	"
	.25	4.7 ± .2	4.5 ± .3	"
l-Tryptophane	1	6.9 ± .2	5.1 ± .2	26
	3	6.1 ± .4	1.6 ± .3	73
l-Cystine	.5‡	4.7 ± .2	.1 ± .1	98
l-Isoleucine	2.5	4.2 ± .1	3.3 ± .4	21
	5	4.2 ± .1	2.1 ± .1	50
l-Ergothioneine	.4	5.0 ± .1	1.8 ± .3	64
	2.5	5.0 ± .1	.1 ±0	100
d,l-3,4-Dihydroxyphenylalanine	.5	4.7 ± .2	1.4 ± .1	71
	1.2	5.0 ± .1	1.1 ±0	77
	2.5	4.2 ± .1	.4 ± .2	91
Indole-3-acetic acid	1	4.2 ± .1	1.5 ± .2	64
	2	4.2 ± .1	1.1 ± .1	74
Casein hydrolysate (Amigen)	3 mg/ml	4.7 ± .2	2.6 ± .1	46
	6.3 "	5.0 ± .1	.4 ± .1	92

* Figures in this column consist of percentage radioactivity in TCA supernatant after incubating insulin-I^{131} with the enzyme preparation, after subtracting the control value which consisted of amount of supernatant radioactivity in beakers containing only insulin-I^{131} and buffer; the latter value usually was less than 1%. Figures shown are avg of 3 determinations; maximal deviation is shown by each.

† Conditions relative to the figures in this column are like those in the preceding paragraph except that test inhibitor was added to control and enzyme-containing beakers.

‡ In order to keep this compound in solution it was necessary to maintain a pH of 8.2; controls for this determination were also at pH 8.2.

NSD = No significant difference.

mechanisms must be considered. It may be hypothesized that cystine protects insulin from degradation by inactivating the enzyme system by oxidation of the sulfhydryl groups. The mechanism by which the other compounds are inhibitory is not known. It is noteworthy that dihydroxyphenylalanine is very active while tyrosine, with a structure differing only in the absence of a hydroxyl group, is inactive.

In comparable investigations, to be reported in detail subsequently, we have found inhibition of insulin degradation by the following compounds: thiamine, thioctic acid, indole, skatole, phenylacetic acid, phenylpropionic acid and indole-3-propionic acid.

Studies are being conducted in mice and man to determine to what extent the effective inhibitors augment the hypoglycemic action of insulin.

Summary. In an *in vitro* system using insulin-I^{131} and a special liver preparation which degrades insulin, it has been shown that certain amino acids and related compounds inhibit insulin-I^{131} degradation, as measured by the accumulation of trichloracetic acid soluble radioactivity. Marked inhibition was demonstrated using cystine, ergothioneine, 3, 4-dihydroxyphenylalanine, isoleucine, tryptophane, indole-3-acetic acid, and casein hydrolysate (Amigen). In the concentrations tested no significant inhibition was exerted by glutamine, glycine, β-alanine, asparagine, arginine, serine, threonine, leucine, methionine, histidine, hydroxyproline or tyrosine.

The authors are very grateful to Drs. Henry H. Tomizawa, Yadviga D. Halsey and Hiromichi T. Narahara for their helpful suggestions.

1. Shmidt, A. A., and Saatchian, R. L., *Zuhr. Eksp. Biol. i. Med.,* 1929, v11, 42.

2. Mirsky, I. A., and Broh-Kahn, R. H., *Arch. Biochem.,* 1949, v20, 1.

3. Tomizawa, H. H., Nutley, M. L., Narahara, H. T., and Williams, R. H., *J. Biol. Chem.,* 1955, v214, 285.

4. Mirsky, I. A., Perisutti, G., and Dixon, F. J., PROC. SOC. EXP. BIOL. AND MED., 1954, v86, 228.

5. Tomizawa, H. H., and Williams, R. H., *J. Biol. Chem.,* 1955, v217, 685.

6. Tyberghein, J., Tomizawa, H. H., and Williams, R. H., *J. Biol. Chem.,* in press.

7. Frankel, H., and Fuchs, J., *Deutsche Med. Woch.,* 1955, v80, 1449.

8. Mirsky, I. A., *Ciba Foundation Colloquia on Endocrinology, Boston,* 1953, v6, 263.

9. Mellinkoff, S. M., Frankland, A. B., and Greipel, M. Presented at the meeting of the Western Society for Clinical Research, Carmel, Jan. 27, 1956.

10. Williams, R. H., *Metabolism,* 1956, March.

Received April 3, 1956. P.S.E.B.M., 1956, v92.

Anti-Inflammatory Activities of Several 9 α-Halo Derivatives of Adrenal Steroids.* (22372)

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The synthesis of a series of 9 α-halo derivatives of adrenal steroids has been reported by Fried and co-workers(1-3). These compounds have been shown to possess unusually high adrenocortical activity in the rat(3-5), in the dog(6,7), and in man(7,8). Several of these compounds are part of a series prepared to study the possible influence of variations in side chain upon adrenocortical action. In the present paper the anti-inflammatory activities of these compounds are reported, using as the anti-inflammatory index the inhibition of formation of granulation tissue around a cotton pellet.

The use of cotton as an irritant in anti-inflammatory studies has been previously reported(9,10). Inhibition of the inflammatory reaction to cotton by either systemic or local administration of cortisone has been demonstrated by Meier, Schuler and Desaulles(9).

Materials and methods. A 5 to 7 mg cotton pellet is inserted high into the upper dorsal area of a male rat (weight range 120-150 g) at the time of bilateral adrenalectomy. The pellet is prepared from non-sterile unbleached cotton, which is rolled into a ball with the fingers(11). The pellet is inserted with long thin forceps, subcutaneously,

* Preliminary report presented at Fed. Am. Soc. for Exp. Biol., San Francisco, April 1955, (*Fed. Proc.,* 1955, v14, 281).

TABLE I. Granuloma Formation in Adrenalectomized Male Rats Treated with Adrenocortical Steroids and Some of Their 9α Halo Derivatives.

Compounds	Total dose (mg), 4-day period	No. of animals	Mean granuloma dry wt (mg)	Potency (12, E or F acetate = 1)	95% confidence limits (12)
Controls	—	20	25.5		
E acetate	1.2	19	17.0	1.0	
	3.6	20	12.5		
	10.8	18	10.2		
F "	1.2	20	20.4	1.0	
	3.6	20	13.5		
	18.8	19	10.0		
Controls	—	29	25.1		
Fluoro F acetate	.13	19	19.8		
	.4	29	15.1	13.2	8.7–20.0
	1.2	20	7.8		
	3.6	10	5.0		
E acetate	1.2	19	19.8		
	3.6	18	11.1		
	10.8	10	10.9		
F "	1.2	10	30.9		
	3.6	10	19.9		
	10.8	10	14.1		
Controls	—	20	19.7		
Fluoro E acetate	.13	20	12.0	10.0	7.1–14.2
	.4	10	7.9		
	1.2	19	4.3		
F acetate	1.2	19	12.9		
	3.6	10	8.1		
	10.8	19	4.7		
Controls	—	20	25.5		
Chloro F acetate	.8	20	14.5	3.4	2.0– 5.9
	2.4	20	6.9		
	7.2	8	4.1		
F acetate	1.2	20	20.4		
	3.6	20	13.5		
	10.8	9	5.4		
Controls	—	19	17.5		
Corticosterone	3.6	8	16.4	.32	.24– .44
	5.4	9	12.0		
	10.8	19	7.1		
F acetate	1.2	19	13.6		
	3.6	20	7.5		
	10.8	20	5.4		
Controls	—	19	15.0		
9α-Fluorocorticosterone acetate	.4	10	14.0		
	.8	8	8.8	2.7	1.8 –4.0
	1.2	10	8.4		
	2.4	9	3.9		
	3.6	10	4.9		
F acetate	1.2	18	12.3		
	3.6	20	7.8		
	10.8	9	4.1		
Controls	—	18	17.0		
9α-Fluoro-11β, 17α-dihydroxyprogesterone	1.2	18	17.8		
	3.6	16	15.3	.37	.16– .85
	10.8	19	9.9		
F acetate	1.2	17	13.6		
	3.6	20	9.8		
	10.8	15	8.7		
Controls	—	18	17.1		
9α-Fluoro-11β, hydroxyprogesterone	1.2	17	12.4		
	3.6	16	14.4	<.1	
	10.8	16	12.7		
F acetate	1.2	17	13.8		
	3.6	20	9.8		
	10.8	15	9.4		
Control	—	10	23.5		
Progesterone	32.4	10	22.5	<.04	
DCA	32.4	10	22.8	"	
F acetate	1.2	10	17.2		
	3.6	8	12.1		
	10.8	10	10.7		

through the incision used for adrenalectomy. Care is taken neither to separate the skin from the connective tissue nor to form a pocket into which the pellet fits. The animal is then injected subcutaneously with an aqueous suspension(4) of the steroid under examination or with the aqueous menstruum (control) and placed on a normal stock diet, with 1% NaCl in the drinking water. On the following 3 days the animal receives once daily an identical injection of the steroid or menstruum. Twenty-four hours after the last injection, corresponding to 96 hours after pellet implantation, the animal is sacrificed and the pellet, along with the granulation tissue that surrounds it, is carefully dissected from the animal and weighed. The dry weight of the granuloma and pellet is obtained after heating at 80°C overnight. By subtracting the initial weight of the pellet, the dry weight of the granuloma is determined. The animals treated with high doses of steroids presented a pellet that appeared by gross inspection in most instances to be almost entirely devoid of granulation tissue. Probably the increase in the dry weight of these pellets is due to an accumulation of adsorbed body fluids. It is reasonable to assume that the solids in these fluids contribute to the weight of the granulomas in the animals treated with the lower doses of steroids or the control menstruum. No attempt has been made to differentiate the weight increments of the granulation tissue from the tissue fluid solids. In the assay results discussed subsequently, the term granuloma applies to the total increase in dry weight of the implanted pellet. Decreasing granuloma weights were observed with increasing doses of active steroids. A linear log dose response has been obtained, using daily levels of 300, 900 and 2700 μg of cortisone acetate (E acetate) or hydrocortisone acetate (F acetate). Occasionally only a minimal response was obtained at the low dose level. Aqueous suspensions of 9 α-fluorohydrocortisone acetate (fluoro F acetate), 9 α-fluorocortisone acetate (fluoro E acetate), 9 α-chlorohydrocortisone acetate (chloro F acetate), desoxycorticosterone acetate (DCA), corticosterone, 9 α-fluorocorticosterone acetate, progesterone, 9 α-fluoro-11 β-hydroxyprogesterone, and 9 α-fluoro-11 β, 17 α-dihydroxyprogesterone have been studied in this manner.

Results. There is no significant difference (12) in the response to E acetate and F acetate in this test. As a result these compounds were assigned a potency of 1 for comparative studies with the other steroids. The results obtained with all of the steroids mentioned above are presented in Table I. It can be seen from the data that there is a decrease in granuloma formation as the dosage of the steroid is increased. The greatest response was obtained with the fluoro derivatives, fluoro F acetate and fluoro E acetate. An intermediate response was obtained with chloro F acetate and 9 α-fluorocorticosterone acetate. The comparative potencies along with 95% confidence limits as calculated by the method of Bliss(12) are listed in Table I. It can be seen that fluoro F acetate is the most active steroid tested having a potency of 13.2 times that of E or F acetate.

In Table II are summarized the data obtained for all of the 9 α-fluoro-11 β-hydroxy steroids containing various combinations of 17 α- and 21-hydroxyl groups. Corticosterone and F acetate are also included in order to compare the activity of these compounds

TABLE II. The Effect of 9α-Halogenation and Hydroxyl Substitution on the Anti-Inflammatory Activity of Adrenal Steroids.

Compound	Hydroxyl at position	Potency	95% confidence limits(12)
F acetate	11β, 17α, 21	1.0	—
Fluoro-F-acetate	11β, 17α, 21	13.2	8.7 –20.0
Corticosterone	11β, 21	.32	.24– .44
9α-Fluorocorticosterone acetate	11β, 21	2.7	1.8 – 4.0
9α-Fluorodihydroxyprogesterone	11β, 17α	.37	.16– .85
9α-Fluorohydroxyprogesterone	11β	<.10*	—
Progesterone	—	<.04†	—
Desoxycorticosterone acetate	21	" †	—

* No activity observed at doses as high as 2.7 mg/day.

† No activity observed at doses as high as 8.1 mg/day.

with their 9 α-fluoro analogues. It can be seen that introduction of the fluorine atom into the 9 α-position of either F acetate or corticosterone increases the anti-inflammatory activity of these steroids approximately 13 and 9 times, respectively. It is also noted that the 17 α- and 21-hydroxyl groups are required for maximum activity. From a comparison of the data obtained with 9 α-fluoro-11 β, 17 α-dihydroxyprogesterone with 9 α-fluorocorticosterone acetate, it appears that the 21-hydroxyl group has a much greater influence on activity than the 17 α-hydroxyl group. The importance of the 17 α-hydroxyl group, however, is illustrated by the lack of activity of 9 α-fluoro-11 β-hydroxyprogesterone, in which this group is absent. It should be noted that both mono- and dihydroxy derivatives of 9 α-fluoroprogesterone are very insoluble, and the possibility exists that the crystalline suspensions used in this study may not be the optimum formulations for properly evaluating these compounds.

Progesterone, which contains none of the substituents described above is inactive at levels of 8.1 mg per day. DCA, containing only the 21-hydroxyl group, is inactive at 8.1 mg per day. This latter observation adds further confirmation to the concept that 11 β-oxygen is required for the anti-inflammatory activity of corticoids.

Summary. The anti-inflammatory properties of a number of 9 α-halo-11 β-hydroxy steroid derivatives have been compared with some of their halogen-free analogues. The substitution of a fluorine atom at the 9 α-position enhances activity. The presence of various hydroxyl groups in the steroid molecule is essential for maximum anti-inflammatory effect. Hydroxylation at the 17 α-position results in a steroid with increased anti-inflammatory activity. A considerably greater response is demonstrated by the introduction of a 21-hydroxyl group, while maximum activity is achieved by the presence of both 17 α- and 21-hydroxyl groups. The most active compound tested, containing a 9 α-fluorine atom and hydroxyl groups at carbon positions 11 β, 17 α, and 21, was fluoro F acetate. This steroid is approximately 13 times as potent an anti-inflammatory agent as E or F acetate.

1. Fried, J., and Sabo, E., *J. Am. Chem. Soc.*, 1953, v75, 2273.

2. Fried, J., *ibid.*, 1954, v76, 1455.

3. Fried, J., Herz, J. E., Sabo, E. F., Borman, A., Singer, F. M., and Numerof, P., *ibid.*, 1955, v77, 1068.

4. Borman, A., Singer, F. M., and Numerof, P., PROC. SOC. EXP. BIOL. AND MED., 1954, v86, 570.

5. Borman, A., and Singer, F. M., *Fed. Proc.*, 1954, v13, 185.

6. Swingle, W. W., Baker, C., Eisler, M., LeBric, S. J., and Brannick, L. J., PROC. SOC. EXP. BIOL. AND MED., 1955, v88, 193.

7. Liddle, G. W., Pechet, M. M., and Bartter, F. C., *Science*, 1954, v120, 496.

8. Goldfien, A., Thorn, G. W., Beigleman, P. M., and Laidlaw, J. C., *J. Clin. Endo.*, 1954, v14, 782.

9. Meier, R., Schuler, W., and Desaulles, P., *Experientia*, 1950, v6, 469.

10. Meyer, R. K., Stucki, J. C., and Aulsebrook, K. A., PROC. SOC. EXP. BIOL. AND MED., 1953, v84, 624.

11. Dorfman, R. I., personal communication.

12. Bliss, C. I., *The Statistics of Bioassay*, New York, Academic Press, 1952.

Received August 9, 1955. P.S.E.B.M., 1956, v92.

Tolerance in Pure Strain Newborn Mice to Tumor Homografts.* (22373)

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Owen(1) reported that fraternal twin cattle, having a common placental circulation, have as adults 2 blood cell types; their own and that of their twin. Since red blood cells have a limited life span he postulated transfer of red cell precursors between the fetuses, which cells failed to undergo homograft rejection and persisted to propagate red cells of their own immunologic type. Dunsford *et al.* (2) observed a similar phenomenon in humans. On the basis of these observations and certain theoretical considerations, Burnet and Fenner(3,4) predicted that animals exposed to antigens during fetal life would be forever incapable of a specific immunological response to these antigens. However, they were unable to confirm this prediction using the chick embryo as the test animal. Following the lead of Owen, Anderson *et al.*(5) and Billingham *et al.*(6) established that fraternal twin cattle would accept reciprocal skin homografts but would reject skin homografts from siblings, mother, or other twin cattle. Billingham *et al.*(7) produced a similar state in mice, which they defined as "acquired tolerance" to subsequent homotransplantation, by the injection of cells (spleen, kidney, testes) from one inbred strain of mice (prospective donors) into members of another inbred strain (prospective recipients) during fetal life. The latest time in embryonic life at which this state could be produced was a day or two before birth. Injection of cells at or shortly after birth did not regularly establish acquired tolerance and there appeared to be a "null period" at birth during which such foreign cells produced neither "acquired tolerance" nor immunity(8). Woodruff(9) studied this phenomenon in rats and ascertained that in these animals "acquired tolerance" could be produced in the neonatal period up to 2 weeks after parturition. He established that the capacity to develop "acquired tolerance" was not lost abruptly but gradually during the newborn period. Studies by Hanan and Oyama(10), Dixon and Maurer(11), Cinader and Dubert(12), and Smith and Bridges(13) suggest that a similar capacity to develop "acquired tolerance" to simple protein antigens also features the neonatal period in rats and rabbits. It has been established that the rejection of tumor homografts in inbred strains of mice is a function of the genetics of histocompatibility and appears to be identical in mechanism to rejection of normal tissue homografts(14,15). "Acquired tolerance" to lymphoma has been produced by the injection of tumor cells as blood and spleen cells during embryonic life in homologous mice.(20).

It is the purpose of this communication to present observations on the behavior of mammary cancer homografts in inbred strains of mice during the newborn period. We have found it possible to effect successful homotransplantation of strain-specific tumor between each of three highly inbred strains of mice if the tumor transplants are made during the first 3 hours of life.

Material and methods. Mice of the "A", "Z" (C3H) and "Ce" strains were used. Transplantable mammary adenocarcinomas which arose spontaneously in the "Z" (C3H) and "A" strains were selected. The "Z" (C3H) tumor had been retransplanted 55 times and the "A" tumor 41 times, in their respective lines. Each tumor grows only in its specific strain. Fresh tumor suspensions were prepared at 5.0% concentration in saline, using a Potter Elvejhem glass homogenizer. A subcutaneous injection of 0.05 cc was made under the nape of the neck in newborn mice (0-3 hours old) of an homologous strain. In the earlier experiments these sites

* Aided by grants from Minnesota Division of American Cancer Society, and the U.S.P.H.S.

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TABLE I. Growth of Tumor Homografts in Inbred Strains.

Tumor strain	Host strain	No. of mice treated with .05 ml 5% tumor suspension	Mice growing foreign tumor No.	Mice growing foreign tumor %
Z (C3H)	Newborn A	17	9	53
Z (C3H)	Newborn Ce	15	12	80
A	Newborn Z (C3H)	8	8	100
Z (C3H)	Mature A	9	0	0

TABLE II. Histocompatibility Characteristics of "Z" Tumor Grown in "A" Mice.

Strain	No. mice treated with .25 ml 5% "Z" tumor growing in "A" mice	No. taking tumor
A	9	0
Z (C3H)	9	9

leaked. To prevent the possible loss of tumor tissue, subsequent injection sites were covered with collodion. All mice were weaned at one month of age.

Results. The results as tabulated in Table I reveal that the majority of newborn "A" mice accepted "Z" (C3H) tumor. These tumors grew progressively and resulted in death of the animals 1.5 to 2.0 months after injection. At that time lung metastases were invariably present.

Groups of "Z" (C3H) mice injected with "A" tumor and "Ce" mice injected with "Z" (C3H) tumor also accepted the tumor transplants in the neonatal period. On the other hand, attempts at homotransplantation of "Z" (C3H) tumor into mature "A" mice uniformly failed.

To determine whether these tumor homografts retained their histocompatibility characteristics following transplantation and growth in a foreign host, the following experiment was performed. A portion of a "Z" (C3H) tumor growing in a newborn "A" was excised, a 5% tumor suspension prepared and 0.25 cc injected into the right groin of each of 9 adult "A" strain and 9 adult "Z" (C3H) mice. The tumor took in all "Z" (C3H) mice but in none of the "A" mice. This observation demonstrates that the tumor remains a "Z" (C3H) tumor and retains its normal genetic and transplantation characteristics.

Discussion. These experiments show that mammary carcinoma, highly specific for a given strain of mice, may be successfully homotransplanted into an otherwise resistant strain if transplantation is carried out within the first few hours after birth. The mechanism permitting this successful tumor homotransplantation is not yet clear to us, in view of Billingham's failure to produce acquired tolerance to skin homografts in mice by injecting cells from the prospective donor in the newborn period.

It is possible that a degree of tolerance may be achieved by early transplantation, sufficient to permit the tumor to establish itself, pass through a "critical period" and then grow progressively. Woodruff(9) found that some rats accepting initial homotransplants of skin after prior treatment with homologous suspensions of spleen, failed to accept a second homotransplant. These data were interpreted as evidence for the existence of a "critical period"(16). However, the concept of the neonatal period as a "null period" of immunological unresponsiveness may be sufficient to explain our observations. During this "null period" one might anticipate that tumor homotransplants would take, establish themselves, and possess sufficient growth potential to keep a step ahead of the developing immunological forces. A period of immunological unresponsiveness is known to occur in many newborn animals as well as humans (17-19).

A third explanation is that both "acquired tolerance" and the relative immunological unresponsiveness of the newborn have operated to produce the reported results. One might even postulate that the former is a function of the latter.

Summary. Homotransplantation of "Z" (C3H) strain mammary adenocarcinoma to "A" and "Ce" strain mice and "A" strain tumor to "Z" (C3H) strain mice has been accomplished by injecting these tumors within a few hours after birth. After progressive growth in the "A" strain host the "Z" (C3H)

tumors retain their histocompatibility characteristics and can be transplanted into adult "Z" (C3H) strain mice but are rejected by adult mice of the "A" strain.

1. Owen, R. D., *Science,* 1945, v102, 400.

2. Dunsford, I., Bowley, C. C., Hutchison, A. M., Thompson, J. S., Sanger, R., and Race, R. R., *Brit. Med. J.,* 1953, v2, 81.

3. Burnet, F. M., and Fenner, F., *Production of Antibodies.* The Macmillan Co., London, 1949.

4. Burnet, F. M., Stone, J. D., and Edney, M., *Aust. J. Exp. Biol. Med. Sci.,* 1950, v28, 291.

5. Anderson, D., Billingham, R. E., Lampkin, G. H., and Medawar, P. B., *Heredity,* 1951, v5, 379.

6. Billingham, R. E., Lampkin, G. H., Medawar, P. B., and Williams, H. L., *ibid.,* 1952, v6, 201.

7. Billingham, R. E., Brent, L., and Medawar, P. B., *Nature,* 1953, v172, 603.

8. ———, *Ann. N. Y. Acad. Sci.,* 1955, v59, 409.

9. Woodruff, M. F. A., and Simpson, L. O., *Brit. J. Exp. Path.,* 1955, v36, 494.

10. Hanan, R., and Oyama, J., *J. Immunol.,* 1954, v73, 49.

11. Dixon, F. J., and Maurer, P. H., *J. Exp. Med.,* 1955, v101, 245.

12. Cinader, D., and Dubert, J. M., *Brit. J. Exp. Path.,* 1955, v36, 515.

13. Smith, R. T., and Bridges, R. A., unpublished manuscript.

14. Mitchison, N. A., *Nature,* 1953, v171, 267.

15. ———, *J. Exp. Med.,* 1955, v102, 157.

16. Woodruff, M. F. A., *Ann. Royal Coll. Surg. Eng.,* 1954, v11, 173.

17. Freund, J., *J. Immunol.,* 1930, v18, 315.

18. Osborn, J. J., Dancis, J., and Julia, J. F., *Pediat.,* 1952, v9, 736.

19. Good, R. A., Mazzitello, W. F., and Zak, S. J., to be published.

20. Koprowski, H., *Nature,* 1955, v175, 1087.

Received April 3, 1956. P.S.E.B.M., 1956, v92.

Effect of Calcium Disodium Ethylenediamine Tetraacetate on Hypercholesterolemic Rabbits (22374)

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The possible mediation of atherosclerosis by the salts of ethylenediamine tetraacetic acid (EDTA) has been explored recently from the standpoint of its effect on cholesterol metabolism(1,2). Curran has demonstrated also that the incorporation of C-14-carboxyl labeled acetate into cholesterol is increased in surviving liver tissue by the addition of 0.0001 M concentration of both the sodium and calcium forms of EDTA(3). It has been reported by Uhl *et al.* that a high cholesterol diet containing calcium EDTA resulted in lower serum cholesterol levels in rabbits than when the compound was administered as the sodium salt subcutaneously(4). In a recent report a contrary view has been advanced(5). Dietary induced hypercholesterolemia in rats was augmented by the inclusion of EDTA compounds in the diet. Further evidence was presented which indicated that parenterally administered EDTA was without effect on endogenous or exogenous cholesterol metabolism in the rat. The present study was carried out to determine whether parenteral calcium disodium EDTA would influence the course of dietary hypercholesterolemia in rabbits.

Materials and methods. Eight rabbits approximately 6 months old and of both sexes of the New Zealand white albino strain were maintained on a high cholesterol diet and water *ad libitum.* The cholesterol diet was prepared by an adaptation of the method of Wang(6) by dissolving one gram of cholesterol (Fisher) in 5.0 ml of ethyl ether (reagent grade, C. P.) and mixing with 100 g of Gleco rabbit pellets. The animals were fed 100 g of diet per day, 6 days per week. After 11 weeks on the high cholesterol diet, the

* From thesis submitted in partial fulfillment of requirements for Master of Science degree, Department of Chemistry, Georgetown University, Washington, D.C.

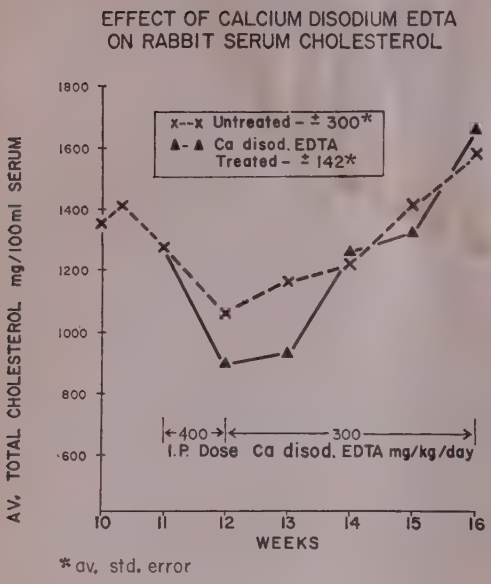

FIG. 1.

animals were divided into experimental and control groups of 4. The experimental group was treated with 400 mg/kg/day of calcium disodium EDTA (Versenes, Inc.) by the intraperitoneal route. After one week the EDTA dosage was decreased to 300 mg/kg/day. Blood for cholesterol analysis was drawn by cardiac puncture. Serum total and free cholesterol levels were determined by the procedure of Zak *et al.*(7). At the cessation of the experiment the animals were sacrificed by air embolism. Aortas and livers were examined for gross evidence of atherosclerosis and fatty infiltration.

Results. The marked hypercholesterolemia of the animals after 11 weeks on the high cholesterol diet is evident by inspection of the data presented in summary in Fig. 1. After one week of treatment with calcium disodium EDTA at a dose level of 400 mg/kg/day the serum cholesterol levels of the treated group decreased sharply. At this dosage level of EDTA the animals were anorexic, their average weight decreased approximately 300 g in comparison to the control group and their food consumption was markedly lowered. When the drug dosage was decreased to 300 mg/kg/day the animals returned to normalcy by the end of one week as indicated by their general behavior and appearance, their increased food intake and their weight recovery toward the level of the control group. The serum cholesterol also returned to the level of the control group after 2 weeks of the reduced EDTA dosage. There were no significant differences in the severity of atherosclerosis and fatty infiltration in the liver as determined by inspection of this limited series of animals.

Discussion. The calcium disodium form of EDTA was selected for this study since in contrast to calcium free sodium EDTA it is without hypocalcemic activity(8). Thus the use of this dosage form of EDTA permitted separation of the metabolic action of EDTA on cholesterol metabolism from its presumed calcium mediating action on the calcium-cholesterol complex. The intraperitoneal route of administration of the chelate avoided the possibility that the known surface active properties of the compound would influence serum cholesterol levels by altering the absorption of dietary cholesterol from the gastrointestinal tract. Further the oral route of chelate administration appeared to rule out the possibility of observation of any direct systemic action of EDTA on cholesterol metabolism since tracer studies have proven the compound to be non-absorbable after oral administration(9).

Results of the present study support the view that EDTA is without effect on cholesterol metabolism. The transient reduction in hypercholesterolemia at toxic levels of the compound may be ascribed to the decrement expected from decreased cholesterol intake in this type of dietary induced hypercholesterolemia. At the maximum tolerated dosage of EDTA there were no significant differences between the serum cholesterol levels of the control and experimental groups.

Summary. Calcium disodium EDTA at the maximum tolerated dosage by the intraperitoneal route had no effect on dietary induced hypercholesterolemia in rabbits.

1. Boyle, A. J., Uhl, H. S. M., and Brown, H. H., *Amer. J. Clin. Path.,* 1953, v23, 801.

2. Perry, H. M., and Schroeder, H. A., *J. Lab. Clin. Med.,* 1955, v46, 416.

3. Curran, G. L., PROC. SOC. EXP. BIOL. AND MED., 1955, v88, 101.
4. Uhl, H. S. M., Brown, H. H., Zlatkis, A., Zak, B., Myers, G. B., and Boyle, A. J., *Am. J. Clin. Path.*, 1953, v23, 1226.
5. Rosenman, R. H., and Smith, M. K., *J. Clin. Invest.*, 1956, v35, 11.
6. Wang, C. I., *J. Mt. Sinai Hosp.*, 1954, v21, 19.
7. Zlatkis, A., Zak, B., and Boyle, A. J., *J. Lab. Clin. Med.*, 1953, v41, 486.
8. Rubin, M., *Metabolic Interrelations*, Josiah Macy Foundation, 1954, v5, 355.
9. Foreman, H., Vier, M., and Magee, M., *J. Biol. Chem.*, 1953, v203, 1045.

Received February 17, 1956. P.S.E.B.M., 1956, v92.

Direct Measurement of Arterial Blood Pressure in the Guinea Pig. (22375)

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Search of the literature for blood pressure values in guinea pigs revealed measurements of mean pressure only(1-3) which are unusually low for small warmblooded mammals (4,5) and which give no indication of the magnitude of diastolic and systolic pressures. In view of the increasing use of this species in research and an expressed need for reference values(6) measurements under as normal conditions as possible were made to fill this gap.

Methods. A Statham P-23D pressure transducer and Sanborn amplifier and recorder were used to obtain records of end pressures in the carotid arteries of 8 NIH or Hartley strain guinea pigs which weighed 200-1000 g. The animals were etherized for exposure of the artery and then maintained for an hour under local or general anesthesia as indicated. The cannula was made from a #20 hypodermic needle and polyethylene tubing (i. d. 0.86 mm) and was 14.3 cm long. The pressures recorded during 1-minute periods at 0, 15, 30, 45 and 60 min. were averaged for each animal. Mean arterial blood pressure was calculated as the sum of diastolic + ⅓ pulse pressure.

Results. Table I shows the average and the range of pressures for each animal. Systolic pressures above 100 mm Hg were unusual and occurred briefly and only during activity. The highest recorded was 140 mm Hg in guinea pig #24. The blood pressure was unusually stable during rapid intravenous injection of saline, 6% dextran or 3.5% polyvinylpyrrolidone in dosages of 1% of the body weight. Under general anesthesia the mean pressure tended to be lower than when the animal was conscious and active, and it gradually decreased as time progressed. The blood pressure changed only momentarily in 2 guinea pigs (#8 and #24) when they were placed in the prone position.

Discussion. Since these measurements were made on occluded arteries, the recorded pressures are slightly elevated over the actual values during flow(7). In general, the average rate of descent of late diastolic pressure fell between that of the rat and rabbit in Table II of Woodbury and Hamilton(8). This was also true of the duration of systole and diastole. However, in 14 of the 33 periods in which it was possible to make measurements, the percentage of the cardiac cycle

TABLE I. Carotid Pressures in Guinea Pigs under General or Local Anesthesia. Average and range in mm Hg.

No.	Anesthesia	Systolic	Diastolic	Mean
17	E, P*	71 (28- 88)	39 (16-48)	50 (22-60)
18	Light E	70 (32- 97)	46 (18-90)	55 (23-87)
19	E, Pr	84 (78-120)	56 (33-76)	66 (25-89)
20	Pr	73 (62- 90)	36 (26-58)	49 (40-61)
21	Pr, E	65 (40-108)	37 (22-69)	46 (30-65)
22	Pr	75 (60- 99)	47 (30-72)	56 (43-81)
23	Pr	92 (56-120)	61 (37-77)	72 (44-90)
24	E, Pr	85 (46-120)	54 (30-74)	64 (36-89)
	Avg	76.7	46.8	57.2

* E = Ether; P = Pentobarb.; Pr = Procaine.

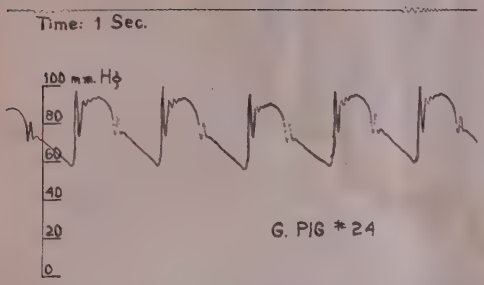

FIG. 1. Record of carotid pressures of a Hartley strain guinea pig weighing about 1000 g under procaine anesthesia, conscious and turned to the prone position 2 min. previously. Mean blood pressure = 68 mm Hg, systolic 88-94, diastolic 55-58, pulse rate 260/min., respiration 64/min., cardiac cycle = 0.232 sec., diastole = 0.118 sec., systole = 49% of cycle, late diastole rate of descent = 179 mm/sec.

taken up by systole (defined as the time from the first upswing of pressure to the bottom of the incisura) was greater than the physiological limit (40-45%) placed by these authors. A retouched record from a conscious prone animal illustrating these points is shown in Fig. 1. The guinea pig heart is therefore capable of faster filling and coronary outflow than was described for other small mammals.

Summary. In 8 guinea pigs under local or general anesthesia, systolic and diastolic end pressures in the carotid artery averaged 77 and 47 mm Hg respectively. The calculated mean pressure averaged 57 mm Hg. The portion of the cardiac cycle taken up by systole was greater than that described for other small mammals in about 40% of the measurements.

1. Porter, W. T., and Richardson, R., *Am. J. Physiol.,* 1908, v23, 131.

2. Landis, E. M., *ibid.,* 1930, v93, 353.

3. Lee, R. E., and Holze, E. A., PROC. SOC. EXP. BIOL. AND MED., 1951, v76, 325.

4. Prosser, C. L., ed., *Comparative Animal Physiology,* 1950, W. B. Saunders Co., Philadelphia, 538.

5. Clark, A. J., *Comparative Physiology of the Heart,* 1927, Cambridge University Press, Cambridge, 114.

6. Reid, M. E., personal communication.

7. Bazett, H. C., and Laplace, L. B., *Am. J. Physiol.,* 1933, v103, 48.

8. Woodbury, R. A., and Hamilton, W. F., *ibid.,* 1937, v119, 663.

Received April 3, 1956. P.S.E.B.M., 1956, v92.

Observations on a Pro-esterase Associated with Partially Purified First Component of Human Complement (C'1).* (22376)

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Investigations on the mechanism of inactivation of human complement by plasmin and by antigen-antibody aggregates(1-5) indicated that the first component of complement (C'1) may exist in serum as enzyme precursor. A mechanism of complement-"fixation" was proposed(3-5) in which it was postulated that antigen-antibody aggregates convert C'1 to active enzyme ("activated C'1"), which in turn inactivates the second and fourth components of complement (C'2 and C'4). Although available experimental data are compatible with this hypothesis, direct evidence has awaited studies with purified systems. Our experiments demonstrate that a partially purified preparation of C'1 converts, under certain conditions of pH, ionic strength and temperature, to material which has lost its hemolytic C'1 activity but

* Presented in part at 128th meeting of Am. Chem. Soc., Minneapolis, Minn., Sept. 14, 1955. This investigation was supported in part by a grant from Lederle Laboratories Division, American Cyanamid Co., and by research grants from the National Heart Institute of National Institutes of Health, Public Health Service and the Cleveland Area Heart Society.

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has gained ability to inactivate complement and to hydrolyze the synthetic ester, p-toluene-sulfonyl-L-arginine methyl ester (TAMe). Disappearance of hemolytically active C'1 occurs simultaneously with appearance of both complement-inactivating and esterase activities. The esterase appears to be distinct from plasmin, thrombin, and acid and alkaline phosphatase.

Nomenclature, materials and methods. Nomenclature, and reagents and methods employed for titration of human *complement* and its components are summarized elsewhere(3, 6-8). Partially purified C'1 loses hemolytic and gains esterase and complement-inactivating activities when adjusted to physiologic pH and ionic strength in the absence of inhibitors; this new product is designated "*converted* C'1" without implying that the esterase and complement-inactivating activities are necessarily derived from hemolytically active C'1. R1 (serum fraction soluble at pH 5.5, ionic strength 0.02, which lacks C'1 but contains C'2, C'3 and C'4) contains inhibitors preventing this apparent inactivation of C'1 and was therefore added in equal amount to the initial dilution in titration of C'1 in purified fractions. Five units of R1 and 1 ml sensitized sheep erythrocytes were then added to 0.2 ml of each dilution and the mixtures incubated at 37°C for 30 minutes(3). *Spontaneous fibrinolytic activity* of preparations of C'1 was measured by a method employing bovine fibrinogen‡ and thrombin(1). To inhibit "conversion" of C'1, the ionic strength of reaction mixtures was adjusted to 0.3. The effect of streptokinase (SK) on fibrinolytic activity was tested by addition of SK to "converted C'1" at ionic strength 0.10 or 0.15 prior to addition of fibrinogen and thrombin; SK was effective in final concentrations of 9 to 500 Christensen(9) units ml. *Caseinolytic activity.* In quadruplicate, 0.3 ml "converted C'1" was mixed with 0.2 ml barbital buffer (0.025 M barbital and 0.125 M sodium chloride at pH 7.5) or of streptokinase (Varidase§) dissolved in buffer in concentration of 10.000 units/ml. These mixtures were incubated at 37°C. 60 minutes. Five-tenths ml 5% casein ("Hammersten quality," Nutritional Biochemicals Co.) was added to 2 tubes at start and to other tubes at end of incubation period. Two ml 0.3 N trichloracetic acid were then added to each tube and the mixture centrifuged. Tyrosine-like activity of the supernatant solution was tested by minor modification of the method of Wu(10). *Digestion of TAMe.* Ability of preparations of C'1 or "converted C'1" to digest p-toluenesulfonyl-L-arginine methyl ester (TAMe) was tested by the method of Troll, Sherry and Wachman(11), modified so that substrate was 0.4 M TAMe dissolved in sodium phosphate buffer (pH 7.5, ionic strength, 0.15). Ionic strength was controlled by altering the concentration of sodium chloride in final mixture. Titration of acid liberated was measured with 1% alcoholic phenolphthalein as indicator. *Digestion of LEe.* Ability of solutions of "converted C'1" to digest L-lysine ethyl ester (LEe) was determined by Sherry's(12) modification of the method of Hestrin(13). Final concentration of LEe in the enzyme-substrate mixture was 0.04 M. Alkaline phosphatase and acid phosphatase activities of 1 ml aliquots of "converted C'1" were determined by the method of Shinowara(14). Thrombic activity was determined at 37° by addition of 0.1 ml of either C'1 or "converted C'1" to 0.3 ml of fibrinogen‖ (300 mg/100 ml of 0.15 M sodium chloride) or to mixture of 0.2 ml fibrinogen and 0.1 ml 0.05 N calcium chloride(15). Ionic strength of the mixtures was controlled by addition of 0.1 ml of sodium chloride in suitable concentration.

Results. 1. *Purification of Human C'1.* A procedure for purification of human C'1 has been reported previously(16). An independent method has been developed which takes advantage of the influence of ionic strength on solubility of C'1 at pH 5.5. The entire fractionation is carried out at pH 5.5 at low temperatures, and C'1 is in the soluble phase only at ionic strength 0.20 or

‡ Kindly provided by Dr. W. H. Seegers.

§ Kindly provided by the Lederle Laboratories Division, American Cyanamid Co.

‖ Kindly provided by the Warner-Chilcott Laboratories.

greater. The importance of this choice of conditions for fractionation will be apparent later. Either fresh human serum or RP may be employed as starting material. RP is serum rendered deficient in properdin by treatment with zymosan at 17° for one hour (17). It offers advantages of being readily available in this laboratory and of greatly reducing the properdin content of partially purified fraction of C'1. However, the method is applicable either to fresh human serum or RP, and the presence or absence of properdin in the final product does not influence reactions to be described.

100 ml serum or RP is dialyzed against pH 5.5 acetate buffer, $\mu = 0.02$, at 1° for 36-48 hrs. The precipitated R2 is recovered by centrifugation at 1°, washed twice with 100 ml of dialysate, and extracted at 1° for 1 hr with 200 ml pH 5.5 acetate buffer, $\mu = 0.12$. The remaining precipitate is re-extracted at 1° for 1 hr with 100 ml pH 5.5 acetate buffer, $\mu = 0.50$. This extract is dialyzed overnight against 2 liter pH 5.5 acetate buffer, $\mu = 0.20$, at 1°. The supernate after centrifugation is brought to 10% methanol at 0° to –2°, and the resulting precipitate suspended in 0.3 M NaCl to a final volume of 5 ml and centrifuged in the Spinco preparative ultracentrifuge at 1°, 100,000 g for 1 hr. The slightly opalescent supernatant, at pH 5.5, $\mu = 0.30$, is stored at –30°.

The final fraction contains 17-25% of the C'1 in the original serum or RP, with a purification of 30-50 fold. Thus, it represents about 0.6% of the original serum protein, which fortuitously coincides with the value found earlier(16). The preparation is heterogeneous in the ultracentrifuge, showing at least 3 components at pH 5.5, ionic strength 0.30.¶

This partially purified fraction of C'1, unlike the previous preparation(16), is not anticomplementary when tested by methods here described. It contains no measurable C'2 or C'4 and only trace amounts of C'3. In preparations concentrated 20 fold over the starting material, the properdin content(17) is about 8 units/ml when prepared from serum and 1 unit/ml or less when prepared from RP. The fraction contains appreciable amounts of streptokinase-activable plasminogen, and the plasmin so activated is fibrinolytic, caseinolytic, and capable of hydrolyzing TAMe and LEe. However, in absence of streptokinase and maintaining the fraction at pH 5.5, ionic strength 0.30, significant spontaneous fibrinolytic, caseinolytic, and esterase activities are *not* demonstrable. Trace amounts of thrombin and perhaps prothrombin are detectable in some preparations. The recently described clotting factor, Hageman factor(18), is a constant contaminant.

2. *Activation of esterase and complement-inactivating properties of partially purified C'1.* The motivation for our experiments was provided by the observation that a solution of partially purified C'1 rapidly loses its hemolytic C'1 activity, when adjusted to pH

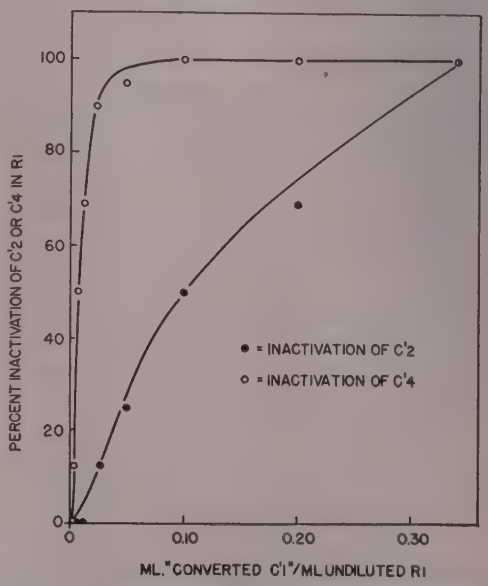

FIG. 1. Inactivation of complement components in human R1 by "converted C'1." "Converted C'1" prepared by diluting partially purified C'1 (5120 units/ml) with equal vol of water to bring to ionic strength 0.15, adjusting the pH to 7.4, and incubating at 37° for 15 min. 0.1 ml of double dilutions of "converted C'1" and 0.15 ml of pH 7.4 barbital-Ca^{++}-Mg^{++} buffer were added to 0.75 ml of 1/1.5 R1 at pH 7.4, ionic strength 0.15, containing 2.5×10^{-3} M Ca^{++}. Samples were incubated at 37° for 30 min. and titrated for complement components. C'3 activity was unaffected and is not shown.

¶ Kindly performed by Dr. M. D. Schoenberg.

TABLE I. Inactivation of Complement-Components in Whole Serum by "Converted C'1."

Serum	"Converted C'1"*	Buffer	Inactivation of complement component†			
	ml		C'1	C'2	C'3	C'4
			%			
1	.02	.48	0	0	0	50
1	.05	.45	0	33	0	95
1	.20	.30	0	50	33	100
1	.50	—	0	67	33	100

* Partially purified C'1 (12,800 units/ml) was diluted with equal vol of water to bring to ionic strength 0.15, adjusted to pH 7.0, and incubated at 37° for 15 min.

† Measured after incubation for 30 min. at 1°, based on complement component titers of serum treated only with 0.5 ml buffer (pH 7.4, barbital-Ca^{++}-Mg^{++}).

7.4, ionic strength 0.15. Concurrently with disappearance of C'1, the preparation becomes highly anticomplementary. This anticomplementary activity of "converted C'1" is a reflection of the ability of the preparation to inactivate C'4 and, to a much smaller degree, C'2. C'3 is not significantly affected. Since plasmin does not inactivate C'2 and C'4 in R1(3), R1 was used to avoid any inactivation of complement which might be due to activation of plasminogen. A typical experiment employing R1 is shown in Fig. 1, in which the reactions were performed at 37°. However, the complement-inactivating property of "converted C'1" is also demonstrable both at 1° and in whole serum (Table I). It will be noted that again C'4 is much more susceptible to inactivation than C'2, and that C'3 is affected little or not at all. It will also be noted that C'1 in serum is not inactivated. The pattern of events is also qualitatively the same when whole serum is incubated at 37° for 30 minutes with "converted C'1."

"Conversion" of C'1 is dependent on time, pH, ionic strength, and temperature. When partially purified C'1 is incubated at 37° for 15 min. at pH 7.4, "conversion" is complete at ionic strength 0.15, only 50% complete at ionic strength 0.20, and is entirely prevented at ionic strength 0.25. At a single ionic strength, 0.15, and at pH 7.4, "conversion" is essentially instantaneous at 37° but requires about one hour at 10° and several hours at 1°. The kinetics of this reaction and of the complement-inactivating properties of "converted C'1" will be presented elsewhere. Use of R1 as test reagent for the assay of complement-inactivating properties of "converted C'1" indirectly precludes the role of plasmin in these reactions(3). The finding that fibrinolytic and caseinolytic activities are absent both before and after the "conversion" reaction directly precludes this possibility. Furthermore, the same amount of streptokinase-activable plasminogen is present before and after "conversion." "Conversion" of C'1 is attended not only by appearance of

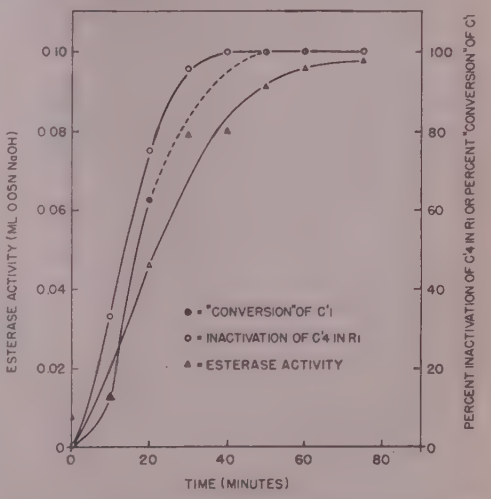

FIG. 2. Disappearance of hemolytic C'1 activity during "conversion" correlated with appearance of esterase and C'4-inactivating properties. Partially purified C'1 (7680 units/ml) was diluted with equal vol of water at 1° to bring to ionic strength 0.15. pH was adjusted rapidly to 7.1 (pH measured at 25° on a separate aliquot) and the sample placed immediately in 10° bath. Aliquots were withdrawn at indicated time intervals and assayed immediately. Hemolytic C'1 was determined as described in this report. The broken portion of the curve represents probable course of reaction, as estimated from the titrations. Marked anticomplementary activity in this region did not permit recording of actual C'1 titers. Esterase activity was determined by adding 0.25 ml of C'1 aliquots to 0.5 ml of 0.4 M TAMe, 1.25 ml of phosphate buffer, and 0.5 ml of 0.8 M sodium chloride. The reaction mixtures, at pH 7.4 and ionic strength 0.30, were incubated at 37° for 1 hr and titrated in usual manner. C'4-inactivating activity was determined by adding 0.01 ml of C'1 aliquots to 0.75 ml of 1/1.5 R1 at pH 7.4, ionic strength 0.15, containing 2.5×10^{-3} M Ca^{++}, and 0.24 ml of pH 7.4 barbital-Ca^{++}-Mg^{++} buffer. Samples were incubated at 1° for 30 min. and C'4 titrated in the usual manner.

complement-inactivating activity but also of esterase activity. The ability to hydrolyze the synthetic ester, p-toluenesulfonyl-L-arginine methyl ester (TAMe), is acquired during the "conversion" reaction. Within limits of experimental error, appearance of esterase activity parallels appearance of complement-inactivating activity and can be further correlated with disappearance of hemolytic C'1 activity. These relationships are shown in Fig. 2.

The esterase is not appreciably active against L-lysine ethyl ester (LEe). In this respect, it again differs from plasmin, which hydrolyzes both TAMe and LEe(11), but resembles thrombin, which hydrolyzes TAMe but not LEe(19). However, the esterase activity of "converted C'1" is apparently not ascribable to thrombin. Thrombic activity is either undetectable or present in only trace amounts in purified preparations of C'1 and no increase is observed following "conversion." Furthermore, purified bovine thrombin‡ which has been diluted to contain the same clotting activity as may be present in partially purified C'1 is inactive against TAMe.

No measurable acid or alkaline phosphatase** activities are present in preparations of "converted C'1".

Discussion. It has been shown with a partially purified preparation of human C'1 that a correlation exists between disappearance of hemolytic C'1 activity, which takes place under certain conditions of pH, ionic strength, and temperature, and appearance of esterase and complement-inactivating properties. The esterase appears to be distinct from plasmin, thrombin, and acid and alkaline phosphatases.

The complement-inactivating activity is directed primarily against C'4. C'1 and C'3 are not appreciably affected. It is not clear whether C'2 is actually inactivated. Disappearance of this component may be an artefact of the method of assay since it takes place only in the presence of very large amounts of "converted C'1". Under these conditions, "converted C'1" may vitiate the assay of C'2 by destroying sufficient *C'4* in the test mixture to prevent immune hemolysis. Additional investigation of this possibility is required.

Esterase activity of "converted C'1" has thus far been observed only with the substrate, TAMe. Studies with other synthetic esters, in addition to LEe which is not appreciably hydrolyzed, are of obvious interest and are in progress.

The earlier proposal that C'1 may be a pro-enzyme(3-5) serves as one possible interpretation of the observations reported. The pro-esterase here described would then be identical with hemolytically active C'1, and the esterase would be identical with the complement-inactivating activity of "converted C'1". However, evidence for such an explanation is still indirect: 1) a good correlation exists between C'1 disappearance and the nearly-simultaneous appearance of both esterase and complement-inactivating activities, as shown in Figure 2; 2) the disappearance of C'1 in partially purified C'1 is probably not due to digestion by the esterase, since the esterase is inert toward C'1 in whole serum (Table I); 3) the known esterase-inhibitor, diisopropylfluorophosphate (DFP), has been reported to inhibit immune hemolysis by guinea pig complement(20,21). The precise relationship of C'1 to the pro-esterase described above remains to be determined by direct experimentation.

Summary. 1. A procedure for partial purification of human C'1 is described. 2. Under certain conditions of pH, ionic strength, and temperature, partially purified C'1 loses its hemolytic activity and gains complement-inactivating and esterase properties. This preparation is designated "converted C'1". 3. Complement - inactivating activity of "converted C'1" is directed primarily against C'4; esterase activity is demonstrable using TAMe as substrate. 4. A correlation exists between disappearance of hemolytically active C'1 during "conversion" reaction and the parallel appearance of complement-inactivating and esterase properties. 5. The possible role of C'1 as a pro-esterase is discussed.

** Kindly performed by Dr. J. W. Price.

Thanks are due to Miss Joan E. Colopy and Mrs. Livia Blum for technical assistance.

1. Pillemer, L., Ratnoff, O. D., Blum, L., and Lepow, I. H., *J. Exp. Med.*, 1953, v97, 573.

2. Lepow, I. H., Pillemer, L., and Ratnoff, O. D., *ibid.*, 1953, v98, 277.

3. Lepow, I. H., Wurz, L., Ratnoff, O. D., and Pillemer, L., *J. Immunol.*, 1954, v73, 146.

4. Lepow, I. H., and Pillemer, L., *ibid.*, 1955, v75, 63.

5. Lepow, I. H., Abst. 128th Meet. Am. Chem. Soc., 1955, p43c.

6. Bier, O. G., Leyton, G., Mayer, M. M., and Heidelberger, M., *J. Exp. Med.*, 1945, v81, 449.

7. Ecker, E. E., Pillemer, L., and Seifter, S., *J. Immunol.*, 1943, v47, 181.

8. Kabat, E. A., and Mayer, M. M., *Experimental Immunochemistry*, Charles C. Thomas, Springfield, Ill., 1948.

9. Christensen, L. R., *J. Clin. Invest.*, 1949, v28, 163.

10. Ratnoff, O. D., and Menzie, C., *J. Lab. Clin. Med.*, 1951, v37, 316.

11. Troll, W., Sherry, S., and Wachman, J., *J. Biol. Chem.*, 1954, v208, 85.

12. Sherry, S., personal communication.

13. Hestrin, S., *J. Biol. Chem.*, 1949, v180, 249.

14. Shinowara, G. Y., Jones, L. M., and Reinhart, H. L., *ibid.*, 1941, v142, 921.

15. Ratnoff, O. D., and Potts, A. M., *J. Clin. Invest.*, 1954, v33, 206.

16. Pillemer, L., Seifter, S., San Clemente, C. L., and Ecker, E. E., *J. Immunol.*, 1943, v47, 205.

17. Pillemer, L., Blum, L., Lepow, I. H., Ross, O. A., Todd, E. W., and Wardlaw, A. C., *Science*, 1954, v120, 279.

18. Ratnoff, O. D., and Colopy, J. E., *J. Clin. Invest.*, 1955, v34, 602.

19. Sherry, S., and Troll, W., *J. Biol. Chem.*, 1954, v108, 95.

20. Levine, L., *Biochem. et Biophs. Acta*, 1955, v18, 28.

21. Becker, E. L., *Nature*, 1955, v176, 1077.

Received January 26, 1956. P.S.E.B.M., 1956, v92.

Polioviruses in Tissue Cultures of Human Conjunctiva and Kidney Cells.* (22377)

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(With the technical assistance of Walter B. Flagg, Salina B. Dwight, and S. Robert Wilson.)

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In the search for a stable strain of tissue culture cells which could be propagated serially on glass, and which could be substituted for monkey kidney cells in mass production of polioviruses, the presumably normal human cell lines established by Chang(1,2) were studied.

To determine if 2 of Chang's cell lines derived from conjunctiva and kidney could support the multiplication of the polioviruses, growth curve experiments were carried out in bottle cultures of these cells and compared to control growth curves in similar cultures of first generation rhesus monkey kidney cells.

Materials and methods. Technics used in this laboratory for the serial subcultivation of conjunctiva and kidney cells have been reported(3). The Mahoney, MEF-1 and Saukett strains of poliovirus were received from the Connaught Laboratories, Toronto, Canada, and were the 3 monkey kidney tissue culture adapted strains, Types 1, 2 and 3, used in the preparation of Salk vaccine. They were the strain pools 54, 55 and 56 distributed to each of the 28 laboratories participating in the 1954 poliomyelitis vaccine field trial. The Parker strain of Type 1 virus was obtained from Dr. David Bodian(4) in the 52nd monkey kidney tissue culture passage. It received 4 additional rhesus monkey kidney passages in our laboratory. The Cleveland 80-4 strain of Type 1 virus, obtained from Dr. Albert Sabin(5), was isolated in HeLa

* Aided by grant from National Foundation for Infantile Paralysis.

cells from a child with "coryza." It received 3 passages in HeLa cells followed by 2 rapid serial passages and 3 terminal dilution passages in cynomolgus monkey kidney cells. In our laboratory it received 3 additional rhesus monkey kidney passages. The YSK strain of Type 2 virus was obtained from Dr. Albert Sabin(6) in 52nd monkey kidney passage. It was an avirulent variant produced by rapid serial passage and terminal dilution technics in monkey kidney tissue cultures. It received 3 additional rhesus monkey kidney passages in our laboratory. Virus titrations were carried out in roller tubes of rhesus monkey kidney cells using half log dilutions, 4 tubes per dilution, and the infectivity endpoints were calculated by the method of Reed and Muench. Growth curves were obtained in milk dilution and Povitsky bottle cultures of conjunctiva and kidney cells. Both cell types were prepared for the growth curve studies in 2 types of media, (1) Chang's medium (CM) consisting of human serum 20%, Hank's balanced salt solution 75%, chick embryo extract 5%, and crystalline soybean trypsin inhibitor 0.001%, and (2) Eagle's medium (EM) consisting of horse serum 20%, Eagle's medium 80%, and crystalline soybean trypsin inhibitor 0.001%. To both media were added penicillin (100 μ), streptomycin (100 μg) and nystatin (25 μg). Monkey kidney cell cultures were cultivated in a nutrient medium consisting of solution 199 (99.5%) and horse serum (0.5%). Prior to infection cell cultures in milk dilution bottles were washed 5 times with 8 ml of balanced salt solution to remove the serum. On the basis of an estimated 10-fold dilution of serum with each washing the residual serum concentration was less than 1 part in 500,000. The bottles were then inoculated with 1 ml of poliovirus with a titer of $10^{5.9}$ to $^{7.7}$ $TCID_{50}$ per ml. Nine ml of solution 199 was added immediately and a sample removed for titration ("Seed" sample) after which the bottle was incubated one hour at 37°C. The medium was then removed, the culture was washed 10 times with 8 ml of balanced salt solution to remove unabsorbed virus and the bottle was then refed with 10 ml of solution 199. Samples for titration were removed immediately after this washing procedure ("0" hour sample) and at intervals of 1, 4, 8, 24, 48 and 72 hours. Povitsky bottles were washed 10 times with 100 ml of balanced salt solution prior to infection. The bottles were then inoculated with 25 ml of poliovirus. Five hundred ml of Eagle's medium was added immediately and samples for titration were removed at varying intervals ranging from 24 to 138 hours. Growth curves of the Parker, Mahoney, MEF-1 and Saukett strains in trypsinized monolayer cultures of first generation rhesus monkey kidney cells cultivated in milk dilution bottles served as controls.

Results. The results of titrations of 3 separate growth curves of Parker strain carried out in milk dilution cultures of conjunctiva cells are presented in Table I. After the adsorption period and the washing, between $10^{0.83}$ and $10^{1.7}$ $TCID_{50}$ of residual virus were left in the 3 experiments.

The graphic summary of the replicate growth curves of the Parker strain carried out in the 2 cell lines cultivated in CM is presented in Fig. 1. Similar triplicate growth curves of the Mahoney, MEF-1, Saukett, Cleveland and YSK poliovirus strains were essentially identical and could be superimposed on the curves of Fig. 1. For brevity these 30 growth curves are omitted. Fig. 2 shows single growth curves of Parker, MEF-1 and Saukett strains carried out in milk dilution bottle cultures of conjunctiva and kidney cells cultivated in EM. These two cell lines had been cultivated in EM containing 20% horse serum for a period of 4 months and still

TABLE I. Three Growth Curves of Poliovirus Type I, Parker Strain in Conjunctiva Cells. (Chang's medium.)

Time	Reciprocal of log $TCID_{50}$/.25 ml		
	Exp. 1	Exp. 2	Exp. 3
Seed	5.25	5.25	5.7
0 hr	1.75	.83	1.33
1	1.75	1.75	1.7
4	2.33	2.75	2.7
8	5.0	5.33	5.83
24	5.67	6.7	6.75
48	6.7	7.2	6.75
72	7.17	6.5	7.0

Seed virus at 37°C = $10^{4.33}$ $TCID_{50}$ after 72 hr.

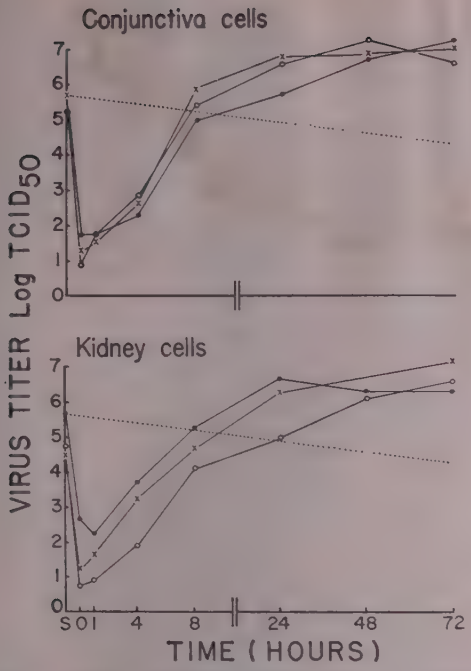

FIG. 1. Three replicate growth curves of Parker strain of Type 1 poliovirus in human conjunctiva cells and human kidney cells cultivated in milk dilution bottles in Chang's medium with 20% human serum. The dotted line represents seed virus incubated at 37°C in the absence of cells. "S" is the sample removed immediately after inoculation and "O" is the sample taken after period of adsorption and washing to remove excess virus.

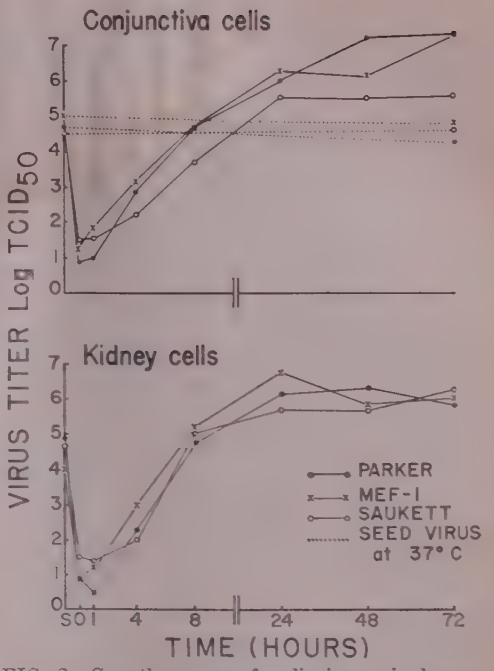

FIG. 2. Growth curves of polioviruses in human conjunctiva and human kidney cells cultivated in milk dilution bottles in Eagle's medium with 20% horse serum.

supported the growth of polioviruses as well as the parent cell lines which had been maintained in CM containing 20% human serum.

Control growth curves of the Parker, Mahoney, MEF-1 and Saukett strains carried out in milk dilution cultures of trypsinized monkey kidney cells are shown in Fig. 3. The maximal virus titers occurred between 48 and 72 hours and were $10^{6.62}$ for the Parker strain, Type 1, $10^{7.75}$ for Mahoney Type 1, $10^{8.0}$ for MEF-1 Type 2, and $10^{7.13}$ for Saukett Type 3. Fig. 4 summarizes the single growth curves of Parker, MEF-1 and Saukett strains carried out in Povitsky bottle cultures of conjunctiva cells cultivated in EM.

Discussion. There is a remarkable similarity between the general appearance and shape of the 49 growth curves described here (involving 6 virus strains, 3 media, 3 cell types and 2 sizes of culture bottles). The 3 replicate growth curves of the Parker strain shown in Fig. 1 indicate the reproducibility of the curves as well as the range of variation of the

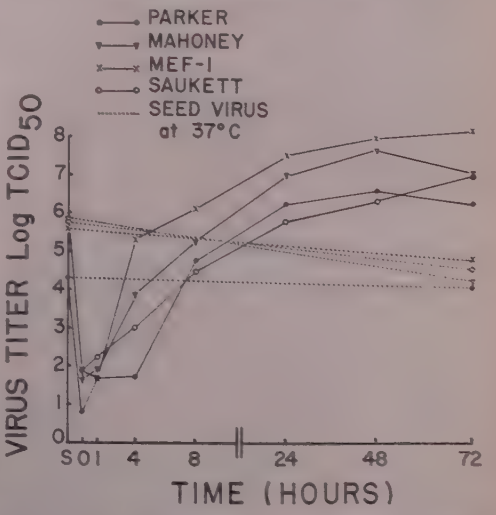

FIG. 3. Growth curves of polioviruses in monkey kidney cells cultivated in milk dilution bottles in medium 199 (99.5%) and horse serum (0.5%).

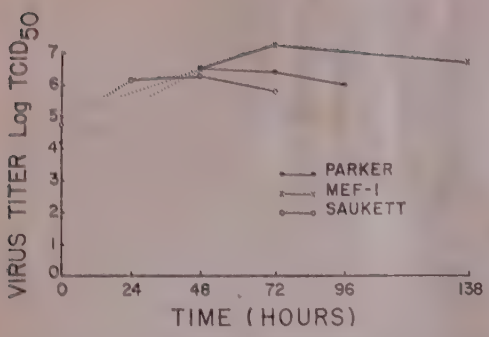

FIG. 4. Growth curves of polioviruses in human conjunctiva cells cultivated in Povitsky bottles in Eagle's medium with 20% horse serum.

method. There is usually a significant release of new virus by 4 hours. During the next 4 hours the virus release is very rapid and 20% of the curves reach the maximal titer by 24 hours, 65% by 48 hours. During the 72 hour period of observation there is no diminution of titer as a rule. No significant differences were observed between the 2 human cell strains or between these and monkey kidney cells. The yield of virus was approximately $10^{7.0}$ $TCID_{50}$/0.25 ml for all 6 viruses. This compares favorably with the virus titers of the Mahoney, MEF-1 and Saukett strains used in preparation of Salk vaccine for the 1954 trials where Povitsky bottle cultures of monkey kidney cells yielded virus pools with titers of $10^{5.5}$ to $10^{7.0}$ $TCID_{50}$(7).

Growth curves of poliomyelitis viruses obtained in monkey testis and kidney and strain HeLa have been described by Ledenko(8), Farrell(9), Melnick(10), Dulbecco(11), Lwoff(12), Scherer(13) and Ackerman(14).

The human conjunctiva and kidney cell lines as carried in our laboratory appear to satisfy all of the desirable technical criteria for mass production of polioviruses. They are easily grown on glass and can be passed serially in mass cultures. They can be carried in series in the absence of all human serum and retain their ability to support the proliferation of polioviruses. The relatively simple medium devised by Eagle or Parker's medium(199) with added horse or calf serum are adequate for prolonged cultivation. These sera can be removed by washing without damage to the cells. Finally, the growth curves of 6 poliovirus strains representing all 3 types give virus yields equal to those obtained in monkey kidney cells. The uniformity of the growth curves for all 6 strains suggests that the cells would support in a similar manner growth of other strains. These experimental growth curves have been obtained in mass cultures rather than on a test tube scale and the Povitsky bottles represent the container size which has been used for commercial mass production of poliovirus.

Summary. Tissue cultures of Chang's human kidney and human conjunctiva cells carried *in vitro* for 17 and 20 months, respectively, were tested for their ability to support growth of polioviruses. Three Type 1 strains, 2 Type 2 strains and 1 Type 3 strain were studied. Growth curves in the 2 human cell lines were compared with growth curves in first generation rhesus monkey kidney cells. The yield of viruses and the shapes of the growth curves were similar for all 3 tissue cultures. Human conjunctiva cells and human kidney cells which were cultivated 4 months in the presence of horse serum instead of human serum retained their ability to support the proliferation of polioviruses.

1. Chang, R. S., PROC. SOC. EXP. BIOL. AND MED., 1954, v87, 440.

2. ———, *Trans. N. Y. Acad. Sc.,* 1955, v17, 584.

3. McAllister, R. M., and Coriell, L. L., PROC. SOC. EXP. BIOL. AND MED., 1956, v91, 389.

4. Bodian, D., personal communication.

5. Sabin, A., personal communication.

6. Sabin, A., Hennessen, W., and Winsser, J., *J. Exp. Med.,* 1954, v99, 551.

7. Salk, J. E., Lewis, J. L., Bennett, B. L., Ward, E. N., Krech, U., Youngner, J. S., and Bazeley, P. L., *Am. J. Public Health,* 1955, v45, 151.

8. Ledenko, N. J., Riordan, J. T., and Melnick, J. L., PROC. SOC. EXP. BIOL. AND MED., 1951, v78, 83.

9. Farrell, L. N., Wood, W., Franklin, A. E., Shimada, F. T., Macmorine, H. G., and Rhoades, A. J., *Canad. J. Public Health,* 1953, v44, 273.

10. Melnick, J. L., *Ann. N. Y. Acad. Sci.,* 1955, v61, 734.

11. Dulbecco, R., and Vogt, M., *ibid.,* 1955, v61, 790.

12. Lwoff, A., Dulbecco, R., Vogt, M., and Lwoff, M., *ibid.,* 1955, v61, 801.

13. Scherer, W. F., Syverton, J. T., and Gey, G. O., *J. Exp. Med.*, 1953, v97, 695.
14. Ackerman, W. W., Rabson, A., and Kurtz, H., *ibid.*, 1954, v100, 437.

Received February 10, 1956. P.S.E.B.M., 1956, v92.

Effect of Ethionine on Blood and Depot Lipids in Experimental Nephrotic Hyperlipemia.* (22378)

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The pathogenesis of nephrotic hyperlipemia is unclarified. An experimental approach to that problem has been facilitated since it was shown(1) that the renal disease induced in rats by the injection of anti-kidney sera obtained from rabbits simulates the nephrotic syndrome as observed in infants and children. Feinberg(2) and co-workers recently have shown that the oral administration of dl-ethionine markedly reduced the concentration of fatty acids, phospholipids and cholesterol in the blood of normal dogs. It thus seemed of interest to investigate the effect of this agent on the hyperlipemia that is regularly associated with the experimentally induced nephrotic syndrome in rats.

Procedures. Methods used to induce the nephrotic syndrome in rats have been described(1). All animals were kept in single cages, were fed Friskies and had unrestricted access to water. Determination of carcass and liver fat was carried out in rats that were fasted for 16-18 hours. They were exsanguinated from the heart under ether anesthesia. The liver fat concentration was determined by the method described by Payne (3) on approximately 1 g of liver tissue. The gastrointestinal tract was freed of its content by washing with saline solution. The entire carcass was then put through a meat grinder, dried at 100°C to constant weight (4-6 days) and ground to homogeneity with mortar and pestle. Three aliquot samples weighing approximately 1.5 g were placed in screw-cap bottles, covered with 50 cc petroleum ether and shaken intermittently for 48 hours. These were filtered under vacuum in fritted glass funnels lined with filter paper. The procedure was repeated until the filtrate was colorless. If not clear, the filtrate was centrifuged before evaporation and the fat residue was determined gravimetrically. 3.5 to 7.5 mg of dl-ethionine‡ per 100 g rat was given to 28 nephrotic rats by intraperitoneal injection twice daily for 5-21 days. A 2% saline solution of ethionine in 0.9% NaCl was used. Three additional rats were treated for 5 days with 13 mg ethionine and 12 mg methionine per 100 g rat per day. These agents were given in one syringe and their concentration was approximately equimolar.

Results. The effect of ethionine on blood lipids was studied in 12 nephrotic rats. A precipitous decline of the markedly increased total lipid and cholesterol values to normal or subnormal values was noted in all animals that received 11 mg or more of ethionine per 100 g rat per day (Fig. 1). When 7-10 mg was given, this effect was less regularly observed. The decrease occurred during the first 3-5 days of ethionine administration and subsided within a few days after its administration was discontinued. This effect was noted in male as well as in female rats. No other effect on the nephrotic renal disease was noted. Proteinuria, blood pressure and further course of the disease were not affected, and the microscopic appearance of the renal lesions was not different from that in

* This study has been supported by a grant from the Cleveland Area Heart Society.

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‡ U. S. Industrial Chemicals Co., Division of National Distillers Corp., New York.

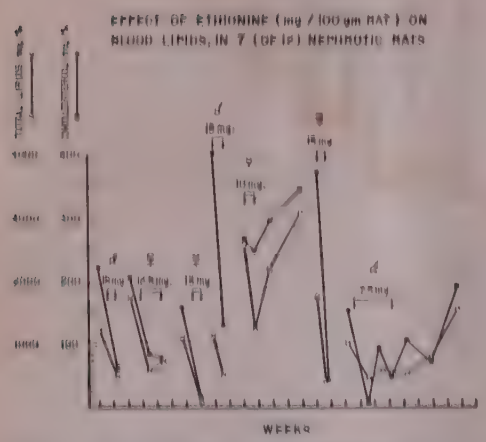

FIG. 1. Only the first 7 of 12 observations are reproduced. Connected arrows indicate duration of daily administration of ethionine.

nephrotic rats that were not given ethionine.

The amount of fat in the liver and the remaining carcass was determined in 28 untreated nephrotic rats, in 16 nephrotic rats that were treated with ethionine, and in 17 healthy controls of similar body weight.

The carcass fat values varied between 21 and 49.5 g per 100 g dry carcass. No significant difference between the 3 groups was noted.

Fig. 2 shows that when compared with values obtained in healthy controls, the liver fat is significantly decreased in nephrotic rats (4) ($p = < 0.01$). In nephrotic animals treated with ethionine, this difference is no longer observed. The values for these rats are within the range obtained in control animals. In the nephrotic male rats that were treated with ethionine and dl-methionine,§ the total lipid and cholesterol concentration decreased in their plasma to subnormal values as rapidly as was observed when only ethionine was used. The liver fat values in these 3 rats were within normal range.

Discussion. It has been shown that dl-ethionine produces fatty livers in fasted female rats(5), while little or no change in the lipid content of the liver was noted in fasted male rats(6). It has also been reported that the simultaneous injection of methionine along with ethionine prevents the accumulation of lipids in the liver of the fasted female rat(6). The precipitous decline of hyperlipemic total lipid and cholesterol values in the plasma of nephrotic rats and the simultaneous increase of liver fat from subnormal to normal values in these animals, is neither sex-linked nor prevented by simultaneous injections of methionine. It thus seems probable that a different mechanism of ethionine action may be at play in the healthy, fasted female rat and in the nephrotic animal. The observation that ethionine decreases the concentration of plasma lipids in nephrotic animals to normal or even subnormal values with simultaneous and proportional increases of abnormally low liver fat values again suggests that the liver(7) plays an important role in the pathogenesis of the nephrotic hyperlipemia. This finding, and the observation

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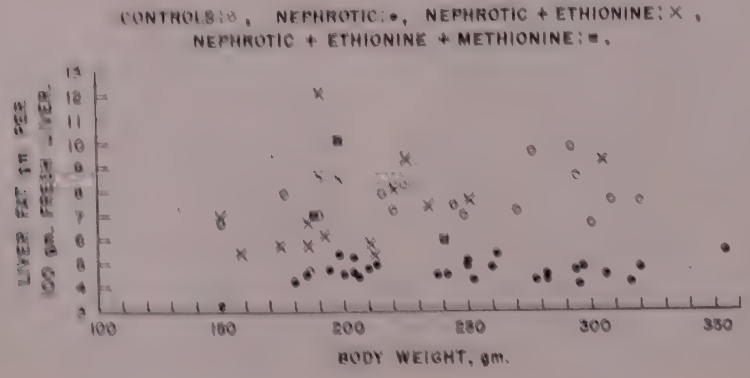

FIG. 2.

that the concentration of fats in the liver of untreated nephrotic animals is significantly diminished when plasma lipids are markedly increased, lends support to the hypothesis (8) that the nephrotic hyperlipemia may be due to an inability of the liver to take up plasma lipids. It has been proposed(9) that ethionine interferes with protein formation. Since practically all serum lipids are present as lipoproteins, it is conceivable that an effect of ethionine on lipoproteins in the liver explains both the increase of liver fat and concomitant decrease of plasma lipids in nephrotic rats.

It is of interest to note that the abolition of the nephrotic hyperlipemia remained without demonstrable effect on the course of the nephrotoxic renal disease in rats.

Summary. 1. Dl-ethionine reduces the markedly increased plasma lipid concentration of nephrotic rats to normal or subnormal values within 3-5 days. 2. The diminished liver fat values regularly noted in nephrotic rats increase to normal levels under ethionine administration while carcass fat values remain unchanged. 3. The effect of ethionine on hyperlipemia and liver fat of nephrotic rats was noted in female as well as male rats, and was not prevented by methionine. 4. These results lend support to the view that the nephrotic hyperlipemia may be due to an inability of the liver to take up plasma lipids normally.

1. Heymann, W., and Lund, H. Z., *Pediatrics*, 1951, v7, 691.

2. Feinberg, H., Rubin, L., Hill, R., Enterman, C., and Chaikoff, I. L., *Science*, 1954, v120, 318.

3. Payne, R. W., *Endocrinology*, 1949, v45, 505.

4. Heymann, W., and Hackel, D. B., to be published.

5. Farber, E., Simpson, M. V., and Tarver, H., *J. Biol. Chem.*, 1950, v182, 91.

6. Jensen, D., Chaikoff, I. L., and Tarver, H., *ibid.*, 1951, v192, 395.

7. Heymann, W., and Hackel, D. B., *Metabolism*, 1955, v4, 258.

8. Rosenman, R. H., Friedman, M., and Byers, S. O., *J. Clin. Invest.*, 1955, v34, 700.

9. Simpson, M. V., Farber, E., and Tarver, H., *J. Biol. Chem.*, 1950, v182, 81.

Received December 2, 1955. P.S.E.B.M., 1956, v92.

Effect of Pre-Treatment of $P^{32}O_4$ Solutions on Uptake and Release of P^{32} by Erythrocytes.* (22379)

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The presence of non-orthophosphate contaminants in $P^{32}O_4$ solutions has been recognized by several investigators. Causey and Harris(1), studying the uptake of $P^{32}O_4$ by skeletal muscle, attributed some of their erratic results to strongly adsorbable trace substances, and suggested that evaporation to dryness with acid reduced the interference by these compounds. Rouser and Neuman detected impurities in Oak Ridge $P^{32}O_4$ preparations, and recommended heating with nitric acid(2). Sato *et al.*(3) subsequently showed that these entities could be separated from orthophosphate by electrochromatography. In connection with studies on the release of phosphate from erythrocytes we were interested in learning whether we would have to use the rather tedious electrochromatographic separation to obtain a homogeneous preparation of orthoradiophosphate, or whether acid-evaporation would serve. A comparison of the two procedures in release studies gave results which prompted us to carry out further experiments on the uptake of phosphate by erythrocytes.

Methods. In the release studies, erythro-

* Work performed under the auspices of the U. S. Atomic Energy Commission.

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cytes from healthy adult mongrel dogs were used. To a 45 ml portion of heparinized blood, obtained by jugular puncture, were added 20 μc $H_3P^{32}O_4$, 0.2 millimole pH 7.4 phosphate buffer, and 0.25 millimole of glucose. This blood was aerated at 37°C for 2 hours. The cells were then collected by centrifugation at 3000 rpm for 10 minutes at 2°C, washed twice with fresh plasma, and resuspended in plasma. Aliquots were transferred to Warburg flasks, gassed with O_2:CO_2 or N_2:CO_2 (95:5), and placed in a water bath at 37°C where they were shaken constantly at 96 oscillations per minute. After equilibration for 10 minutes, and at frequent intervals thereafter up to one hour, samples of plasma were prepared for counting by pipetting aliquots onto aluminum discs. Radioactivity was measured on dried samples with a Model PCC-10 proportional counter (Nuclear Measurement Corporation). Those taken after the 10 minute equilibration were designated as the time-zero samples, and the amount of radioactivity present in these was subtracted from subsequent counts. Uptake studies were carried out with rabbit blood. A flask containing a 10 ml sample of heparinized blood was gassed with O_2:CO_2 and equilibrated at 31°C for 30 minutes, with constant stirring. One-tenth ml of $P^{32}O_4$ solution (2 μc) was added. Ten minutes later, and at frequent intervals thereafter, 0.7 ml of blood was withdrawn, and the plasma prepared and counted as described above. Two flasks, containing different preparations of $P^{32}O_4$, were used in each experiment. $P^{32}O_4$ solution obtained from Oak Ridge was pre-treated in 3 ways. The first consisted of evaporation to dryness on a hot plate (ca. 270°C) with HNO_3 and H_2O_2. Electrochromatography plus radioautographic analysis demonstrated that the material thus treated contained an entity which did not migrate with the orthophosphate component. The second pre-treatment consisted of an electrochromatographic separation by the method of Sato *et al.*(3). The zone containing orthophosphate was located on the filter paper by radioautography. The orthophosphate was then eluted, and in order to remove the slight amount of paper fibers, lactic acid, and metallic ions found in the eluate, treated again with HNO_3 and H_2O_2 (but not taken to complete dryness, so that the temperature of the sample was never above *ca.* 100°C), and passed through a cation exchange resin (Dowex 50). The homogeneity of this material was confirmed by radioautography. Three different batches were used in these studies. The third preparation consisted of a sample of electrochromatographically homogeneous $P^{32}O_4$ which was evaporated to dryness and heated for 30 minutes in a sand bath at 300°C, then resuspended in 0.05 N HCl. This treatment results in formation of significant amounts of polyphosphates(4). These pre-treated solutions are thus identified as (I) acid-evaporated only, (II) electrochromatographed, and (III) electrochromatographed and evaporated.

Results. The data obtained on release of phosphate in 3 experiments with electrochromatographed $P^{32}O_4$ and in 2 with acid-evaporated material are shown in Fig. 1. It is apparent that the release of phosphate was about twice as fast in those experiments in which the electrochromatographed material

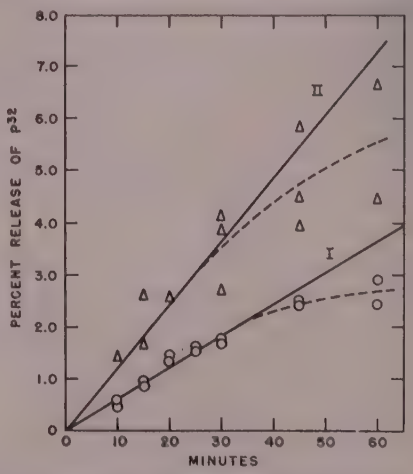

FIG. 1. Release of phosphate under aerobic conditions from dog erythrocytes previously incubated with (I) acid-evaporated and (II) electrochromatographed P^{32}. Each point is an avg of duplicate determinations. The solid lines are the slopes calculated by the method of least squares using values obtained for the first 30 min. The values are for the electrochromatographed, .123%/min., S.E. .0077; and for the acid-evaporated, .061%/min., S.E. .0018; $p < .001$.

was employed. Fig. 2 shows that the inhibition observed aerobically was noted under anaerobic conditions as well. Other experiments have been carried out at different temperatures, and similar effects have been observed; these experiments will be discussed in detail elsewhere.

The results of 3 experiments in which uptake of phosphate was measured are shown in Fig. 3. It is clear that the treatment employed here produced an inhibitory substance which decreased the initial rate of uptake by nearly 60%. As in the case of the release experiments, studies at other temperatures have invariably confirmed the observation that phosphate uptake is diminished when evaporated $P^{32}O_4$ solutions are used.

Discussion. These data emphasize that the pre-treatment given to radioactive phosphate solutions prior to use in biological systems can drastically influence experimental results. Evaporation with HNO_3–H_2O_2 either fails to remove a pre-existing inhibitor, or else produces an inhibitory substance. The latter explanation is seemingly favored by the fact that evaporation of electrochromatographically homogeneous $P^{32}O_4$ effected an inhibition in phosphate uptake, although the former possibility is of course not excluded. That the electrochromatographic treatment may have produced an activator which could be destroyed by subsequent evaporation seems highly unlikely, since the presumably "activated" samples are homogeneous while the "normal" ones (*i.e.*, acid-evaporated) are not.

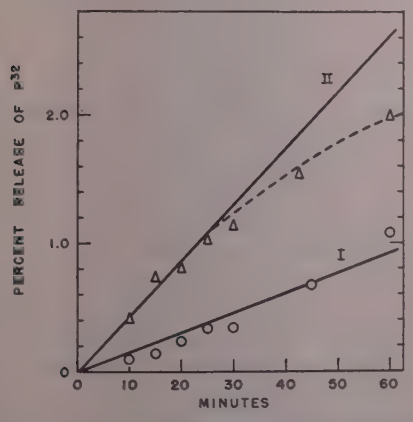

FIG. 2. Release of phosphate under anaerobic conditions, same preparations as above. The slope of II is .0439%/min., S.E. .0016; of I, .0154%/min., S.E. .0012; $p < .001$.

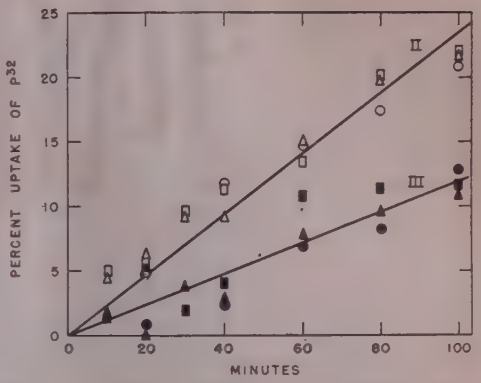

FIG. 3. Uptake of phosphate by rabbit erythrocytes incubated with (II) electrochromatographed and (III) electrochromatographed and evaporated P^{32}. The symbols show the individual experiments in which II (open symbols) and III (closed symbols) were used simultaneously. The slope of II is .234%/min., S.E. .0095; of III, .120 %/min., S.E. .0066; $p < .001$.

We have had little experience with systems other than those described here. However, one of us (A. O.) has noted that the uptake of radioactivity into the acid-soluble fraction of the isolated rabbit heart was diminished by 50% when acid-evaporated $P^{32}O_4$ was added to the perfusion fluid.

Our findings suggest that all samples of $P^{32}O_4$ be examined for homogeneity, and if evidence of heterogeneity is found the samples should be purified before use in a biological system. The electrochromatographic separation is a rather time-consuming process but appears to be a highly effective one.

Summary. The release of phosphate from dog erythrocytes was about twice as fast when blood had been previously incubated with electrochromatographed orthophosphate rather than with $P^{32}O_4$ which had been acid-evaporated without previous electrochromatographic treatment. Similar inhibitory effects were observed in the uptake of phosphate by rabbit erythrocytes when an electrochromatographed phosphate solution was evaporated to dryness prior to use.

We wish to thank T. R. Sato and W. E. Kisieleski

for their advice and assistance in the electrochromatographic and acid-evaporation technics. We are also indebted to S. A. Tyler for advice on statistical treatment of the data.

1. Causey, G., and Harris, E. J., *Biochem. J.*, 1951, v49, 176.

2. Rouser, G., and Neuman, W. F., *Fed. Proc.*, 1952, v11, 278.

3. Sato, T. R., Kisieleski, W. E., Norris, W. P., and Strain, H. H., *Anal. Chem.*, 1953, v25, 438.

4. Sato, T. R., Kisieleski, W. E., Norris, W. P., Smetana, F., and Strain, H. H., manuscript in preparation.

Received December 15, 1955. P.S.E.B.M., 1956, v92.

A Strain of Unclassified Cells Isolated from Lymph Node of Patient with "Reticulo-Endotheliosis".* (22380)

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Strains of human cells *in vitro* have been found useful in cytologic and virus investigations; interest has centered mainly on epithelial-like cells either of carcinomatous origin(1,2) or from normal tissues(3), although it is well known that human fibroblasts can be subcultured for long periods of time(4). The present report describes a strain of cells derived from a lymph node biopsy of a patient with the clinical diagnosis of "reticulo-endotheliosis" and now subcultivated for 20 passages (7 months).

Clinical Data. The patient from whom this strain of cells was isolated, a 50-year-old male, noted in 1943 the gradual onset of a generalized reddish-brown, maculo-papular rash which eventually covered his entire body, except his face, and which has persisted until the present time. In May 1951, he developed symptoms of epigastric distress and weight loss; at this time positive physical findings included enlarged axillary lymph nodes and splenomegaly to the umbilicus. Peripheral blood counts were normal and biopsies of a skin lesion and lymph node revealed "nonspecific inflammatory changes." X-ray therapy (225 r) to the spleen at this time produced no change in the patient's condition. Three years later the patient noted persistent diarrhea and, in Dec. 1954, presented the typical picture of decompensated cirrhosis with muscle wasting, marked hepato-splenomegaly and ascites. Laparotomy revealed marked mesenteric lymphadenopathy with areas of infiltration into the bowel wall, liver and spleen. Pathologic studies of a mesenteric node, liver, bone marrow and skin revealed infiltration by clumps of cells which were interpreted as reticulo-endothelial cells. Since May 1955, the patient has shown a dramatic response to ACTH therapy including cessation of diarrhea, clearing of ascites, marked reduction in the size of the liver and spleen, improvement in skin lesions and disappearance of the generalized lymphadenopathy.

Materials and methods. Tissue culture methods employed were those now in wide use(5,6). A cervical lymph node biopsy was performed 6 days after start of daily ACTH therapy. Half of the node was placed in fixative for pathologic examination and half placed in synthetic medium(7), hereafter referred to as MS, for transport to the laboratory. The node was minced and placed in roller tubes both in a plasma clot and directly on the glass. The nutrient medium consisted of MS 74%, horse serum 20%, human serum 5%, chick embryo extract 1% and antibiotics, either penicillin 50 units per ml and streptomycin 50 μg per ml or tetracycline 25 μg per ml. For virus work the human se-

* This work was supported in part by research grants from the National Foundation of Infantile Paralysis and from the Research Laboratories of Chas. Pfizer Co.

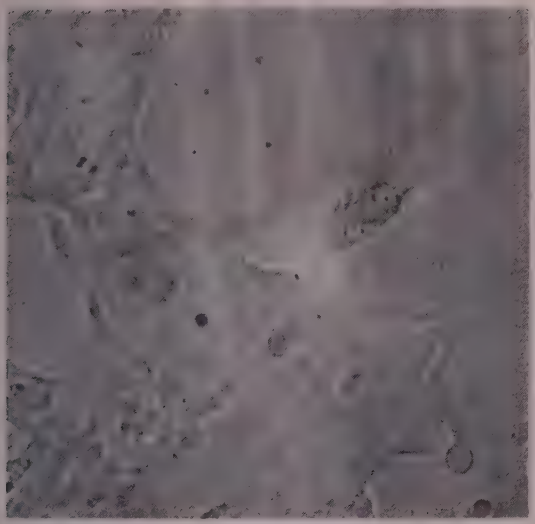

FIG. 1. Roller tube culture of LN cells. Unstained; 63×.

rum was omitted. Sera were inactivated at 56°C for 30 minutes and came from human and equine sources. Acid phosphatase activity was detected by the method of Gomori(8) and the periodic acid-Schiff procedure as described in his textbook(9).†

Initial Isolation. Fibroblastic outgrowths were noted in all tubes by 4th day and in some cultures groups of cells, probably macrophages, were seen. In certain cultures the latter appeared eventually to predominate and many unsuccessful attempts were made to separate them from the fibroblasts. On 29th day of cultivation, outgrowth in 3 tubes was pooled and subcultured. One of these subcultures showed marked proliferation of macrophages and thereafter frequent partial transfers were made from this source. In one such attempt, performed after this culture had been maintained for 81 days, a different cell type was observed. By scraping from the glass and transplanting, these cells, designated the LN strain, were eventually obtained in independent cultures.

The LN strain. A small group of cells, epithelial-like in appearance, was first observed on 10th day following subculture from a tube containing predominantly macrophages. At first these cells formed a monolayer of polyhedral cells, but after subcultivation the cells tended to form small clumps several layers thick. Their characteristics have not apparently changed during 20 passages (7 months). If allowed to grow, these clumps quickly detached and occasionally began to grow elsewhere in the bottle or tube. The cells could be scraped off the surface with ease, usually coming off the glass in small clumps which could easily be broken up into very small groups of cells or into single cells by pipetting; occasionally the cells could be simply washed from the glass surface. Growth of these cells is rapid following subcultivation in medium containing human serum, somewhat slower without this ingredient. Although these cells can be subcultured by the use of trypsin(6), it was early found that they were easily and satisfactorily subcultivated by scraping and pipetting. When observed early in their growth period through glass or in stained preparations, the cells look not unlike the HeLa strain(1) or any of several epithelial-like strains derived from normal human tissues(3). (Fig. 1).

Susceptibility to Cytopathogenic Agents. Because of their morphologic appearance and source, it was thought likely that these cells

† These procedures were carried out in the Department of Anatomy, Harvard Medical School, through the courtesy of Dr. Leon P. Weiss.

TABLE I. Titration of Poliomyelitis Virus, Type I, in Human Liver and LN Cells.

Virus log dilution	Cell strain	Time in hr 0	22	30	48	56	72	96	120
-1	L	—	4+						
	LN	—	4+						
-2	L	—	4+						
	LN	—	3+	3+	4+				
-3	L	—	2+	4+					
	LN	—	—	1+	3+	3+	4+		
-4	L	—	—	2+	4+				
	LN	—	—	1+	3+	3+	4+		
-5	L	—	—	—	2+	3+	4+		
	LN	—	—	—	1+	2+	3+	4+	
-6	L	—	—	—	—	—	—	—	—
	LN	—	—	—	—	—	—	—	—
-2 + immune serum	L	—	—	—	—	—	—	—	—
	LN	—	—	—	—	—	—	—	—

L = Liver epithelial cells(3). LN = LN strain from lymph node of patient with "Reticulo-endotheliosis."

— = No cytopathogenic effect, cells normal. 1+ = 1-5 groups of cells showing cytopathogenic effect. 2+ = 6-20 groups of cells showing cytopathogenic effect. 3+ = Most cells show cytopathogenic effect, but normal appearing cells still present. 4+ = Cytopathogenic effect complete, no normal cells remain.

would respond to poliomyelitis virus in a manner similar to other human cell lines; some differences have been observed. The LN cells are destroyed more slowly than the strains of normal epithelial-like cells maintained in this laboratory(3). Table I presents data from one of three experiments in which this time relationship has been consistent.

Histochemical Procedures. Since macrophages are known to exhibit considerable acid phosphatase activity(10) and since the LN strain appeared to originate from this type of tissue, parallel cultures of liver epithelium and LN cells were stained on coverslips by the calcium phosphate method at pH 4.6. The liver cells were well stained but even macroscopically it could be seen that the LN cells were far more heavily stained, indicating a high degree of acid phosphatase activity. Essentially similar findings were obtained when the two lines were stained by the periodic acid-Schiff procedure, indicating a high concentration of complex saccharide or lipid substances containing potential aldehydes. These studies are being continued.

Summary. A strain of human cells isolated from the lymph node of a patient under treatment with ACTH with the clinical and pathologic diagnosis of "reticulo-endotheliosis" is reported. This cell line can be subcultured readily after 20 passages (7 months) and, although morphologically similar to the human epithelial-like strains now established, differs from them with respect to its source, certain growth characteristics, and in its response to the cytopathogenic action of poliomyelitis virus. The cells show striking acid phosphatase activity and stain heavily by the periodic acid-Schiff procedure.

1. Gey, G. O., Coffman, W. D., and Kubicek, M. T., *Cancer Research,* 1952, v12, 264.

2. Berman, L., Stulberg, C. S., and Ruddle, F. H., *Blood,* 1955, v10, 896.

3. Chang, R. S., PROC. SOC. EXP. BIOL. AND MED., 1954, v87, 440.

4. Swim, H. E., and Parker, R. F., *ibid.,* 1955, v83, 577.

5. Weller, T. H., Enders, J. F., Robbins, F. C., and Stoddard, M. B., *J. Immun.,* 1952, v69, 645.

6. Scherer, W. F., Syverton, M. D., and Gey, G. O., *J. Exp. Med.,* 1953, v97, 695.

7. Scherer, W. F., *Am. J. Path.,* 1953, v29, 113.

8. Gomori, G., PROC. SOC. EXP. BIOL. AND MED., 1939, v42, 23.

9. ———, *Microscopic Histochemistry,* University of Chicago Press, Chicago, 1952.

10. ———, *Arch. Path.,* 1941, v32, 184.

Received December 16, 1955. P.S.E.B.M., 1956, v92.

Protection of Heart Under Hypothermia with Acetylcholine Arrest.* (22381)

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Hypothermia will protect specific areas of the body or the total body during periods of circulatory arrest(1,2), and most investigations have emphasized cerebral protection during this period of anoxia. The heart has been protected by the depressed metabolism of the cooled state, but formidable complications continue, and ventricular fibrillation has been considered a major deterrent to the use of hypothermia(3,4). Considering the profound environmental changes associated with hypothermia the heart is remarkably non-sensitive. This is especially true when a relatively normal acid-base relationship is maintained(5,6). However, with circulatory arrest and continuation of cardiac contractions the heart becomes progressively more cyanotic and weaker; there is electrocardiographic evidence of deterioration; and resuscitation becomes progressively more difficult.

This study was based on the thesis that arrest or inhibition of cardiac activity would afford increased protection of the heart. It has been found that a near total arrest can be obtained with the use of intracoronary acetylcholine and this effect of the acetylcholine can be reversed by atropine given shortly before release of occlusion.

Method. Fifteen adult mongrel dogs were used in this study (3 had had prior implantation of yttrium^{90} oxide pellets in the myocardium and were sacrificed for study). Preoperative medication was 45 mg of morphine sulphate only. Four received barbiturates for induction, and all animals had ether-oxygen closed circuit anesthesia with a carbon dioxide absorber in the system. Three dogs were studied at normal body temperature and 12 were cooled to 24-27°C for the experimental period. Cooling and rewarming procedures were done with the use of a Thermo-Rite circulating fluid blanket. Body temperature was observed continuously with the use of a rectal thermocouple and gauge. Lead II of the electrocardiogram was used as the monitor throughout the procedure. Standard and augmented limb lead electrocardiograms were obtained at various stages of the procedure. Total circulatory arrest was obtained by ligating the azygos vein, placing bulldog clamps on the superior and inferior vena cavae and cross clamping the aorta and pulmonary artery with a large Satinsky clamp. Respiration was halted during the periods of occlusion. Except for a period when the chest was open and a short time thereafter, the dogs were allowed to breathe normally. When respiration was assisted at the low temperature, the respiratory rate was maintained at 3 per minute. In 9 of the experiments a constant recording CO_2 analyzer was attached to the endotracheal tube at the level of the mouth and records of inspiratory and expiratory CO_2 were obtained. This indicated a level of expiratory CO_2 to be maintained by the hypoventilation during the time the chest was open. Direct blood pressure readings (via a femoral catheter and a Statham transducer) were recorded on a Grass direct-writer electromanometer. Complete inflow and outflow occlusion was accomplished and maintained for 1 minute and the "occlusion" rate obtained. After stabilization requiring 2 to 4 minutes the heart was again completely occluded from the circulation, respiration stopped, and 100 mg of acetylcholine chloride in 6 ml 154 mM saline was injected into the aorta proximal to the aortic clamp so that the solution went into the coronary system. (The first 4 dogs received only 50 mg of acetylcholine and some of the dogs received an additional 100 mg at 5 minutes.) Circulatory occlusion was maintained for 1 to 3 minutes in the normothermic animals and

* This study supported by grants from the Simms and Douglas Smith Foundations for Medical Research.

TABLE I. Effect of Temperature, Occlusion and Acetylcholine on Cardiac Rate in Dogs.

A. Hypothermia

Temp., °C	Cardiac rate/min. Normothermic	Hypothermic	Occlusion	Occlusion & acetylcholine	Return	Acetylcholine Total time, min.	Acetylcholine No. total contractions	Result
25	142	80	90	5	75	10	51	Sacrificed
24.8	180	70	73	9	75	9	91	"
24.1	170	80	60	6	70	10	58	"
25	220	74	80	3.6	80	9	33	*
24.2	170	65	55	2.5	70	10	25	Living
25	150	70	70	2.3	106	10	23	*
24.5	180	55	90	1	52	10	12	*
26	192	80	88	5	49	10	46	Died post-op.
26.3	175	60	65	13	90	10	132	*
26.8	155	74	80	8	123	9	70	Died post-op.
24.8	90	60	61	15	94	10	147	" "
24.8	140	85	85	14	85	10	158	Living
Ave.	164	70	75	7	81			

B. Normothermia

Temp., °C	Cardiac rate/min. Initial	Occlusion (1 min.)	Acetylcholine & occlusion 1st min.	2nd min.	3rd min.
38	180	155	0	46	88
38.7	150	200	0	9	24
38.5	115	136	7	38	79

* Died of postoperative pneumothorax.

for 9 to 12 minutes in the hypothermic ones. Atropine sulfate (0.6 mg in 5 ml 154 mM saline) was given intracoronary 9 to 10 minutes after the acetylcholine (except in Dog 594). This was forced into the coronary system by manual compression of the heart, and by maintaining occlusion for one minute. Initially only the superior vena cava and aortic clamps were removed, the inferior cava being opened shortly thereafter as filling of the heart was decreasing. Cardiac rate during the acetylcholine to atropine time is recorded, and not the total occlusion period rate for the "acetylcholine" rate. Shortly after a normal sinus rhythm was obtained the "return" cardiac rate was determined.

Results. Table IA summarizes the data obtained on the animals undergoing hypothermia. Hypothermia *per se* produced a considerable decrease in the pulse rate. It is most significant that occlusion under these controlled conditions produced little change in the cardiac rate, 7 increased, 2 decreased and 3 remained unchanged.

Acetylcholine effected a profound and instantaneous reduction in cardiac rate. The contractions during the period of acetylcholine effect were of ventricular origin. The auricle showed no contractions in most preparations but in a few there was auricular flutter. Following injection of atropine and reinstitution of circulation, sinus rhythm and preocclusion rates were rapidly obtained.

The usual pattern for cardiac recovery was: 1. auricular flutter with a variable ventricular rhythm, 2. various lessening degrees of partial heart block and 3. normal sinus rhythm. This was usually accomplished within 2 to 3 minutes, occasionally within 30 seconds. The ventricular contractions from the beginning were of good quality and produced adequate blood pressure (with the exception of dog 124). Throughout the procedure the heart remained pink and appeared far more normal than a similarly occluded, but unprotected heart. Withholding the atropine (for 15 minutes post occlusion in Dog #594) showed a prolonged period of heart block with sinus rhythm returning 2 minutes after the atropine was given.

The usual pattern of the electrocardiogram through the various phases of temperature

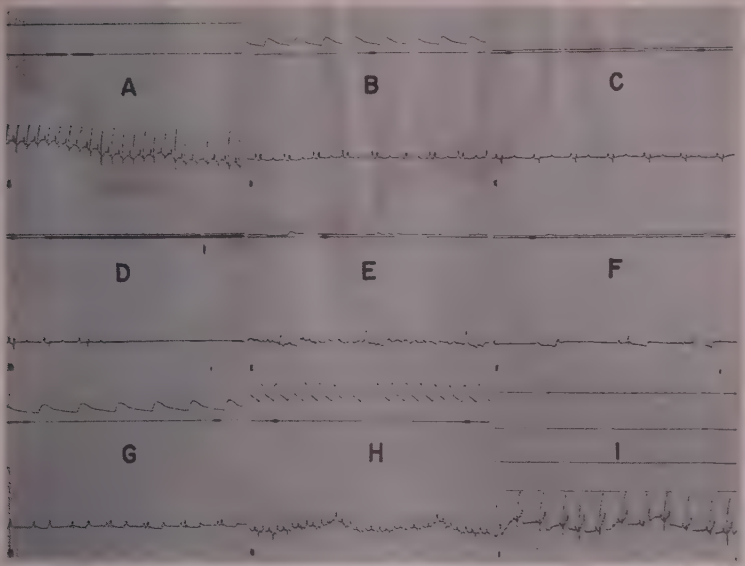

FIG. 1. Lead II electrocardiogram and blood pressure recording of Dog #466. (A) Preoperative, (B) hypothermic (25°C), (C) occlusion, (D) occlusion and acetylcholine (100 mg), (E) release of aortic and superior vena cava occlusion following atropine (0.6 mg), (F) reversion to normal sinus rhythm from heart block within 45 sec., (G) normal sinus rhythm with normal blood pressure 2 min. 12 sec. following atropine, (H) rewarming (37°C), and (I) three wk post-operatively.

change, occlusion only, occlusion with acetylcholine, atropine effect and return is shown in Fig. 1.

Two dogs with relatively high cardiac rates during the acetylcholine occlusion period showed a ventricular rhythm with some periodicity and this was not affected by a second injection of intracoronary acetylcholine.

A major complication has been an unusually high incidence of postoperative and post rewarming pneumothorax, and this has been the cause of a number of deaths. Since no atropine or similar drug has been used (because it would block the acetylcholine effect), there have been more tracheobronchial secretions than usual. This permits persistence of any air leaks. With the use of periodic tracheal suction, avoidance of high pressures when inflating and expanding the lungs, and temporary catheter chest drainage, this complication has been largely surmounted.

Three animals were studied under similar circumstances at normal temperatures (Table IB.). Again it was noted that circulatory occlusion alone had no consistent effect on the cardiac rate. Following injection of acetylcholine the heart was stopped in 2 animals and markedly slowed in the third. This effect was short-lived. The rates increased dramatically in the second and third minutes of occlusion and rapidly returned to normal sinus rhythm without the use of atropine.

Discussion. This study is the fruit of a search(6) for a method to protect the occluded heart by slowing or stopping it in a safe and reversible manner. This method has made it far easier to work within the various cardiac chambers and surprisingly simple to work directly with the coronary arteries.

A perusal of the methods of "desensitizing" the hypothermic heart shows that they have one common effect, namely, slowing the cardiac rate. It is probable that the anti-fibrillatory effect of these methods is merely the result of having protected the heart by slowing it during the period of occlusion. Furthermore, the only real success to date with extreme low temperatures in non-hibernating animals has been that of Niazi and Lewis(5) which was associated with an induced arrest at 13-16°C.

Bjork(7) used acetylcholine unsuccessfully

in attempting to stop the locally cooled heart. This failure is probably related to the use of scopolamine prior to surgery.

Conclusions. 1. Total circulatory occlusion alone does not slow the rate of the normothermic or hypothermic heart. 2. Intracoronary acetylcholine will profoundly decrease the cardiac rate in the occluded heart. The duration of this effect is short at normal body temperatures but quite prolonged during hypothermia (24-27°C). 3. Intracoronary atropine readily reverses the acetylcholine effect on the heart. 4. Cardiac slowing or arrest affords an added protection to the hypothermic heart during total circulatory occlusion.

1. Bigelow, W. C., Callaghan, J. C., and Hoppe, J. A., *Ann. Surg.*, 1950, v132, 531.

2. Lewis, F. J., Varco, R. L., and Taufic, M., *Surgery*, 1954, v36, 538.

3. Swan, H., Zeavin, I., Holmes, J. H., and Montgomery, V., *Ann. Surg.*, 1953, v138, 360.

4. Riberi, A., Siderys, H., and Shumacher, H., *ibid.*, 1956, v143, 216.

5. Niazi, S., and Lewis, F. J., *Surgical Forum Am. Coll. of Surgeons*, 1954, p113.

6. Moulder, P. V., Thompson, R. G., and Harrison, R. W., data to be published.

7. Bjork, V. O., *Acta Chir. Scandinav.*, 1948, v96, supp. 137.

Received December 29, 1955. P.S.E.B.M., 1956, v92.

Rectal Administration of Warfarin (Coumadin) Sodium. Sodium [3(2-acetonyl-benzyl)-4 hydroxycoumarin)]. (22382)

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Considerable experience has accrued with the use of warfarin sodium, the salt of 3-substituted - 4 - hydroxycoumarin, administered orally and intravenously in anticoagulant therapy(1-6). The drug is effective when given by either route and produces a significant prolongation of the prothrombin time in 12 to 16 hours, usually reaching therapeutic levels in 20 to 24 hours. A single dose usually reaches and maintains therapeutic levels in anywhere from 1 to 7 days and achieves a peak effect in 2 days. However, there are occasions when it may be desirable to use another route of administration if feasible. Both methods have their limitations, and rectal administration of the drug would be advantageous under certain circumstances. Experience with the rectal administration of bishydroxycoumarin (Dicumarol) has been limited to that of Meyer and Spooner(7). They reported an occasional significant decrease in the coagulability of the blood which was irregular, however; hence the route proved impractical.

Experience with the use of warfarin (Coumadin) sodium suppositories in 23 patients is the basis for this report. The suppositories contained 100 mg in a polyethylene glycol base.*

Methods. Preliminary studies were performed on 3 groups of patients to determine the latent period and the hours required to achieve the therapeutic level of 25 to 17 seconds (20 to 40% of normal). All prothrombin determinations were made by the one-stage method of Quick with the use of Difco thromboplastin (rabbit brain). Prothrombin times were determined for group A patients 12 and 18 hours after receiving suppositories. Group B patients had prothrombin determinations at 24 and 30 hours, and groups C and D patients at 18 and 24 hours. In each group daily prothrombin times were subsequently measured until they returned to normal or the patient was discharged from the hospital. It soon became apparent that the latent period was more than 12 hours and less than 18 hours, and, consequently, most of the patients had initial prothrombin determinations at 18 and 24 hours. The 19 patients comprising ex-

* Supplied by Endo Pharmaceutical Co., Richmond Hill, through the courtesy of Dr. Samuel M. Gordon.

TABLE I. Effect of a Single 100 mg Warfarin Sodium Suppository.

Patient	Age	Sex	Latent period (hr)	Therapeutic range: Hr to achieve	Therapeutic range: Duration (days)	Peak effect (days)	Return to normal (days)
1A	34	♀	18	24	3	2	6
2	41	♀	18	"	3*	3	4*
3	62	♀	?	36	2	3	6*
4B	43	♂	?	24	2	2	4
5	63	♂	?	"	—	—	—
6C	16	♀	24	"	2	2	3*
7	18	♀	18	"	2	2	4*
8	41	♀	18	"	4	2	6
9	49	♀	18	> "	1	2	6
10	54	♀	24	> "	3*	4	4*
11	56	♀	18	"	3	2	4*
12	61	♀	18	> "	1	2	4
13	72	♀	18	"	6	3	8*
14	27	♂	18	"	2	2	4
15	32	♂	18	"	1	1	4
16	32	♂	18	> "	1	2	3*
17	38	♂	18	"	5	3	6*
18	42	♂	18	> "	3	3	4*
19	53	♂	18	18	3	2	6
20D	63	♀	18	24	2	2	?
21	76	♀	18	"	2	2	?
22	48	♂	18	18	3	2	?
23	60	♂	18	"	?	?(2)	?

* These patients were all discharged before the prothrombin times had returned to normal. In patients 2A and 10C prothrombin times were still in the therapeutic range.

perimental groups A, B, and C had diseases ranging from asthma to muscular dystrophy. Three patients had diabetes mellitus. In 6, the primary diagnosis was that of a functional illness. All these patients were in relatively good physical condition and had no evidence of renal or hepatic disease. The group D patients were those in whom a maintenance oral dose of warfarin sodium was subsequently continued. Two of these patients had intracranial thromboses, one a peripheral thrombophlebitis, and one an acute myocardial infarction. No data are available on the time required for the prothrombin times to return to normal in these patients.

Results. The results for the individual patients are shown in Table I.

The latent period was less than 18 hours in most cases. Initial prothrombin determinations were made on the 2 patients in group B at 24 hours, at which time the therapeutic range had already been obtained. Patient 3-A exhibited no alteration in the prothrombin time in 12 and 18 hours; however, when the prothrombin time was next determined at 36 hours, the therapeutic level had been reached. In 2 of the remaining 20 patients there was a 24-hour latent period.

A therapeutic level of 20 to 40% prothrombin was achieved in the majority of patients within 24 hours. In 5 of the 6 patients requiring more than 24 hours to reach this level, the prothrombin concentration was 50% or less. Patients 1-A and 2-A did not have prothrombin determinations at 24 hours; however, their prothrombin times at 18 hours were elevated to such a degree that it may be assumed that a therapeutic level was reached in 24 hours. In 3 patients the therapeutic range was achieved in 18 hours; 2 of these patients were acutely ill. Once reached, the therapeutic level was usually maintained for 2 to 3 days, the same duration as that following a single oral or intravenous dose of warfarin sodium.

The peak effect was obtained in about 2⅓ days. In only one instance (15-C) did it occur within 24 hours. This suggests the advisability of giving the first maintenance dose after 48 hours, provided the prothrombin concentration is not less than 20%.

The data presented do not suggest that age,

sex, or weight has any marked effect on the patient's response to the drug. These patients weighed from 38½ to 100 kg. Three patients (1-A, 9-C, and 19-C) weighed less than 45 kg, and in none of these was the prothrombin time excessively prolonged (maximum prolongation 26 seconds or 19%). It is advised, however, that if the patient weighs less than 45-50 kg the initial dose for routine use should be 1 mg/kg, with earlier adjustments being made in maintenance doses as indicated. Three patients (11-C, 12-C, and 15-C) weighed more than 80 kg and demonstrated a satisfactory response to the drug. The usual precautions against giving large initial doses in the acutely and chronically ill, in the early postoperative period and in the presence of significant liver (including acute congestion) and or renal disease, must be observed with suppositories as well as other modes of administration. Patient 23-D was one in whom more caution in initiating therapy should have been taken. However, patient 22-D had an acute myocardial infarction and associated acute hepatic engorgement without an excessive response to the 100-mg suppository.

Vit. K_1 was administered to 2 patients. In patient 5-B, 50 mg of K_1 (Mephyton®) was given intravenously 30 hours after the suppository (prothrombin time 23 sec.), and a normal prothrombin time was observed 18 hours later. This was done because the patient was to have a bronchoscopic examination the following day. Forty-two hours after patient 23-D received the suppository, the prothrombin time was 80 seconds. Twenty milligrams of Mephyton® administered intravenously resulted in a prothrombin time of 13 seconds (control, 12 seconds) 24 hours later. Interestingly, although the prothrombin concentration was less than 4%, no side effects occurred and microscopic hematuria was not present. In no other instance was the prothrombin concentration brought below 10%; in only 3 other patients was it reduced to less than 15%. Although urinalyses were not routinely done, in no patient was hematuria observed.

We have not attempted to maintain any patient on rectally administered warfarin sodium, since the oral route has proved satisfactory. However, reasonably accurate divisions of the suppositories could be prepared for this purpose.

Discrepancies and technical difficulties occurred in 5 patients, 3 of whom are not included in this series. In 2 of these, defecation occurred soon after the suppository was inserted (1 within 15 minutes; the exact time of the other unknown). The prothrombin time was unaltered in the former patient and showed only a small and transitory change in the other. In the third patient, the suppository was very soft and incompletely inserted; and only a small and transitory prothrombin alteration ensued. In patients 9-C and 19-C a similar incident fortuitously occurred. In these cases, however, time permitted a second trial with a suppository that had been kept cool and firm in the refrigerator until just prior to use. As recorded in the table, the results in these re-trials were satisfactory. Accordingly, therefore, the condition of the rectum and the suppository, prior to the latter's administration, must be considered carefully.

Discussion. It appears that warfarin sodium in a polyethylene glycol base as a rectal suppository is absorbed with sufficient regularity so that its use is a practical method for administering a coumarin anticoagulant to lower the prothrombin to therapeutic levels. It is of particular value, obviously, in the treatment of a patient who is incapable of taking oral medication, for example, after cerebral thrombosis, or when intravenous administration is difficult or undesirable. Warfarin sodium suppositories make a worthwhile addition to our therapeutic armamentarium. It is possible that suppositories of 75 mg and less might be useful in allowing greater flexibility for this route of administration. It is our plan to test these smaller doses when they are made available.

Summary. Experience with rectal use of warfarin sodium in 23 patients has been recorded. In contrast to Dicumarol, the rectal administration of warfarin sodium is consistently effective. If certain precautions are taken, the rectal route appears to be as reliable as the oral or intravenous, and in some

circumstances it may be the route of choice.

We wish to express our thanks to Miss Donna Martens for her technical assistance.

1. Wolff, J. M., Barker, N. W., Gifford, R. W., Jr., and Mann, F. D., *Proc. Mayo Clinic,* 1953, v28, 489.

2. Clatanoff, Dallas V., Triggs, Perry O., and Meyer, Ovid O., *A.M.A. Arch. Int. Med.,* 1954, v94, 213.

3. Shapiro, S., *J. Kansas Med. Soc.,* 1954, v55, 687.

4. Wright, Irving S., *Ann. Int. Med.,* 1955, v43, 942.

5. Clatanoff, Dallas and Meyer, Ovid O., *A.M.A. Arch. Int. Med.,* in press.

6. Pollock, B. E., *J.A.M.A.,* 1955, v159, 1094.

7. Meyer, Ovid O., and Spooner, Maryloo, Proc. Soc. Exp. Biol. and Med., 1943, v54, 88.

Received February 23, 1956. P.S.E.B.M., 1956, v92.

Radiophosphorus Uptake by Normal, Hyperplastic, and Tumorous Mammary Tissues of Mice.* (22383)

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The hyperplastic nodules of the mammary gland of high-cancer strains of mice have been the object of limited investigation over the past 15 years(1). In general, most of the available evidence points to these nodules as potential sites of tumor formation (hence preneoplastic), although it is important to emphasize that there seems to be a "family" of structures of varying morphology, and possibly of varying growth potential, which are described as hyperplastic nodules. As part of a comprehensive study of these nodules in the C3H/He mouse, we have been attempting to characterize them definitively, both morphologically and physiologically. The present study is concerned with an investigation of a general metabolic characteristic of hyperplastic nodules, based upon their uptake of radiophosphate (P^{32}). Phosphate, playing a key role in cellular metabolism, enters a wide variety of compounds within the cell, and to some extent the rate of incorporation of phosphorus may be used as an index of metabolic activity. In general, the rate of accumulation of phosphate is greater in actively metabolizing and growing cells and tissues(2).

The experiments reported here were designed to answer two questions: (a) Can normal, hyperplastic, and tumorous mammary tissues selected from a single mouse on the basis of their morphologic characteristics also be distinguished on the basis of their P^{32} uptake? (b) Can the relative P^{32} uptake of normal, hyperplastic, and tumorous mammary tissues be altered by varying the endocrine state of the mouse? The results obtained allow us to describe the hyperplastic nodule as a structure metabolically intermediate, as indicated by P^{32} uptake, between normal mammary tissue and frank mammary tumor.

Materials and methods. 81 multiparous female mice of the C3H/He strain, 8-11 months of age, were used in these studies. All mice bore spontaneous mammary tumors of various sizes. They were separated into groups and treated as follows (Table I): (1) 12 normal (untreated, non-pregnant); (2) 15 pregnant (14th-18th day); (3) 14 bilaterally ovariectomized (2 weeks before sacrifice); (4) 16 estrogen-treated (implanted with 2-3 mg pellets of estradiol 2 weeks before sacrifice); (5) 12 cortisol-treated (injected every 2 days with 0.5 mg hydrocortisone acetate in saline suspension beginning 2 weeks before sacrifice); (6) 12 androgen-treated (im-

* Aided by Cancer Research Funds of University of California and by grant from Amer. Cancer Soc. (administered by Committee on Growth). We are indebted to Dr. G. K. Hawkins of Schering Corp., Bloomfield, N. J., for estradiol (Progynon) and testosterone (Oreton-F), to Dr. J. R. Beem of Sharp and Dohme, West Point, Pa., for Hydrocortone Acetate, and to Mr. E. B. Barnawell of our Laboratory for careful selection of animals used.

planted with 4-6 mg pellets of testosterone 2 weeks before sacrifice). An additional 16 lactating, untreated, non-tumorous mice were employed to determine the P^{32} uptake of actively secreting mammary tissue. Most of the lactating mice were free of the milk factor to decrease the possibility of selecting hyperplastic nodules as normal lactating tissue. Animals were injected intraperitoneally 7 hours prior to sacrifice with 2 μc of P^{32} (as sodium phosphate dissolved in isotonic KH_2PO_4) per g body weight. Generally 5 samples of each tissue—normal, hyperplastic, and tumorous—were taken from each mouse. Occasionally, because of the small size of the nodules, 2 or 3 nodules comprised a single sample of hyperplastic tissue. The nodules were selected, removed, and dissected as free as possible from surrounding tissue with the aid of a low-power microscope. Only those nodules were selected in which dense clusters of alveoli could be identified. Tumor tissue samples were selected from non-necrotic areas of small palpable tumors. Tissue samples averaged 400 μg (dry weight, unextracted). Five to 10 samples of mammary gland were removed from the lactating mice, and 5 samples of adipose tissue (generally mesometrial) were taken from each of 11 animals among the several groups. Individual samples were placed on previously weighed, small aluminum foil squares, and were dried at 32-37°C for approximately 15 hours. After drying, the samples on the foil squares were weighed to $\pm$ 2 μg on a calibrated quartz-fiber helix microbalance. Fat was then extracted by placing each sample in 1.5 ml of absolute ether-ethanol (1:3) for 11-15 hours. After extraction, the samples were again weighed, and the % loss in weight (% fat) was calculated. The samples were then placed in individual aluminum planchets and counted (1600-6400 counts) in a flow counter, usually on the second day after sacrifice. Whenever any deviation from this routine occurred, counts were corrected for decay. Final values are expressed in counts per min. per 100 μg (dry, fat-extracted weight) of tissue. The paired data within each of the experimental groups were analyzed to determine the significance of the differences between normal, hyperplastic, and tumorous mammary tissue. The standard error of each mean value was also computed for both P^{32} uptake and % loss in weight. While an attempt was made to inject as closely as possible 2 μc P^{32} per g body weight, the measurement of radioactivity of our original P^{32} preparations was not sufficiently accurate to avoid some error. To control partly for this, members of each experimental group were staggered so that animals from more than one experimental group received P^{32} from each of the preparations used. In addition, the variation in the size of the tumors possessed by individual animals introduced some question as to the reliability of the "body weight" as a figure from which to compute total dosage of P^{32} for each animal. For these reasons and because of the presence of variable amounts of adipose tissue, the data do not lend themselves well to comparisons between experimental groups, but only within groups.

Results. Table I gives the P^{32} uptake in counts per min. per 100 μg of water-free, fat-free mammary tissue. It is clear that hyper-

TABLE I. Radiophosphorus Uptake and Fat Content of Mammary Tissues in Various Endocrine States.

Animal group	No. of mice	Counts (P^{32})/min./100 μg† Mammary tissue*			% loss in wt (% fat) Mammary tissue*		
		Normal	Hyperplastic	Tumorous	Normal	Hyperplastic	Tumorous
Normal	12	409 ± 23	548 ± 46	909 ± 70	81.8 ± 2.0	72.3 ± 2.7	13.8 ± 2.1
Pregnant	15	799 ± 51	861 ± 51	1047 ± 74	77.1 ± 2.9	58.9 ± 3.3	17.7 ± 2.5
Overcastrated	14	425 ± 47	644 ± 51	1070 ±100	66.8 ± 8.2	47.0 ± 5.6	10.3 ± 1.0
Estrogen-treated	16	368 ± 27	619 ± 41	801 ± 78	62.0 ± 7.1	42.1 ± 5.1	11.1 ± 1.7
Cortisol-treated	12	347 ± 33	571 ± 48	806 ± 51	87.3 ± 3.1	67.3 ± 4.6	13.1 ± 1.1
Androgen-treated	12	394 ± 42	557 ± 53	970 ±102	66.5 ±10.6	50.7 ± 7.9	10.4 ± 1.3
Lactating	16	965 ± 89			33.2 ± 3.4		

* Mean ± S.E.$_m$ † Values based on dry, fat-free wt.

plastic nodules show a greater uptake than normal mammary tissue and a lower uptake than tumorous mammary tissue (adenocarcinoma). The paired values within each experimental group are all significantly different at the 1% level of confidence, except within the group of pregnant animals where normal (*i.e.*, prelactating) is significantly different from the hyperplastic tissue (*i.e.*, lactating areas) at the 5% level. Examination of the % loss in weight (Table I) reveals a relationship between these paired values inverse to what is seen with P^{32} uptake, *viz.*, the tumorous tissue loses the least weight, the normal tissue the most. By this criterion, as well as by the metabolic criterion of P^{32} uptake, the nodule is seen to occupy an intermediate position in comparison with normal gland and mammary tumor.

Attempts to alter the relative P^{32} uptake of the three mammary tissue types by endocrine manipulations were essentially unsuccessful. However, during pregnancy the P^{32} uptake of both normal and precancerous tissue is greatly increased, and in general the P^{32} uptakes of various mammary tissues show the relation: nonlactating normal $<$ nonlactating hyperplastic nodule $<$ prelactating normal $<$ prelactating hyperplastic nodule $\leqq$ lactating $=$ tumorous.

Discussion. In general, the untreated hyperplastic nodule approaches normal prelactating mammary tissue in its metabolic activity, as judged by P^{32} uptake, and is significantly less active than mammary tumor tissue. That the tumor tissue should show a high P^{32} uptake is not surprising (*cf.* 2). Kenney(3) has shown a differential absorption of P^{32} in human mammary carcinoma, which was about 5 times that of normal breast tissue, and Tuba *et al.*(4) demonstrated a higher Qo_2 of tumorous than of normal mammary tissue in C3H mice.

The fat extracted by the ether-ethanol is of two principal kinds: (1) that forming the bulk of adipose tissue and (2) that found in the intracellular vacuoles and secretion mass which characterize hyperplastic, prelactating, and lactating mammary epithelium. As indicated in Table I, the bulk of normal tissue samples (except in the lactating mouse where mammary adipose tissue is largely replaced by mammary epithelium) and probably the bulk of nodule samples, consist of adipose tissue from which the parenchyma of the mammary gland cannot be separated by dissection. Extracted adipose tissue samples show a high P^{32} uptake, often as high as extracted mammary tumor samples. Hence, the relative P^{32} uptake of normal, hyperplastic, and tumorous tissues is not a reflection of their content of adipose tissue. In fact, the presence of adipose tissue biases the data against the findings reported in Table I. The differences between the P^{32} uptake of the three mammary tissues are highly significant and demonstrate that these tissues are metabolically, as well as morphologically, different.

The several endocrine treatments were designed to alter the levels of steroid hormones known to affect the immature mammary gland. However, the data do not indicate appreciable metabolic changes resulting therefrom in the adult multiparous C3H mouse and are in partial agreement with our unpublished morphologic observations. Nevertheless, the P^{32} uptake of normal and precancerous mammary tissues is altered by the major changes in the endocrine state of the host involved in pregnancy and lactation.

Summary. The hyperplastic nodule of the nonpregnant C3H/He mouse mammary gland shows a radiophosphate (P^{32}) uptake intermediate between that of normal and tumorous mammary tissue. The relative P^{32} incorporation (tumor $>$ hyperplastic nodule $>$ normal) was not appreciably affected by any of a series of endocrine manipulations (ovariectomy; androgen, estrogen, or cortisol administration). However, during the second half of pregnancy, the uptake by both normal and hyperplastic tissues increases.

1. Gardner, W. U., *Cancer Research,* 1942, v2, 476; Huseby, R. A., and Bittner, J. J., *ibid.,* 1946, v6, 240; Jones, E. E., *Acta Unio Internat. Contra Cancrum,* 1951, v7, 263; Mühlbock, O., Tengbergen, W. van E., and van Rijssel, Th. G., *J. Nat. Cancer Inst.,* 1952, v13, 505; Pullinger, B. D., *Brit. J. Cancer,* 1952, v6, 78; Prehn, R. T., *J. Nat. Cancer Inst.,* 1953, v13, 859.

2. Jones, H. B., Chaikoff, I. L., and Lawrence, J. H., *Am. J. Cancer*, 1940, v40, 243.
3. Kenney, J. M., *Cancer Research*, 1942, v2, 130.
4. Tuba, J., Rawlinson, H. E., Shaw, L. G., Fraser, M. S., and Jeske, I., *Canad. J. Med. Sci.*, 1953, v31, 400.

Received March 2, 1956. P.S.E.B.M., 1956, v92.

Hydrolysis of Arginine Esters by Male Accessory Sexual Tissues. (22384)

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During the course of studies on coagulation of rodent semen(1) it was observed that extracts of the coagulating gland of the guinea pig hydrolyzed tosyl-L-arginine methyl ester (TAMe) at very rapid rates. This TAMe hydrolyzing enzyme could be separated from vesiculase, the enzyme responsible for the coagulation of seminal vesicle proteins, by fractionation with ammonium sulfate and with acetone. In this paper it will be shown that certain esters of L-arginine, but not those of a number of other amino acids, are hydrolyzed by extracts of the prostate gland of some species. Factors affecting the activity of this arginine ester hydrolyzing enzyme, and its intracellular and tissue distribution are discussed.

Methods. The esters of free or substituted amino acids were of commercial origin except for L-lysine ethyl ester, which was synthesized according to Werbin and Palm(2). The melting points of all these substances agreed well with those in the literature. Five times recrystallized soy bean trypsin inhibitor was obtained from the Nutritional Biochemical Corporation. Manometric measurements were made with conventional Warburg flasks fitted with one sidearm. Protein was determined spectrophotometrically(3). Dry weights were determined by heating to constant weight at 100°. The hydrolysis of casein and of hemoglobin at pH 7.5 was measured by the methods of Kunitz(4) and Anson(5) respectively. Canine prostatic fluid was obtained from two dogs which had undergone the prostatic isolation operation of Huggins *et al.*(6). The animals were castrated and had received 50 mg of testosterone propionate per day for some months. The fluid was obtained by the administration of pilocarpine(6), centrifuged to remove small amounts of debris, and stored in the frozen state until used. The accessory sexual organs of various rodents were dissected carefully in order to avoid contamination by secretions of neighboring tissues. We are grateful to Dr. Evelina Ortiz for dissection of the accessories of the mouse and the hamster. In all animals the secretion of the seminal vesicles was removed by expression. The hydrolysis of free or substituted amino acid esters was followed by manometric determination of the carbon dioxide liberated from 0.026 M sodium bicarbonate buffer in equilibrium with 95% nitrogen-5% carbon dioxide at 38°. The reaction was initiated by the addition of the substrate from the sidearm after a temperature equilibration of 10 minutes. In every case suitable blank vessels were set up to correct for any nonenzymatic hydrolysis of the substrate, and also for any acid formation by the tissue extract. All values are given in terms of micromoles of carbon dioxide liberated by the hydrolysis of a given substrate. With crude saline extracts of the coagulating gland of the guinea pig, the rate of hydrolysis of TAMe was strictly proportional to the amount of extract added and to the time of incubation provided that the activity was not greater than 30 μmoles per hour.

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Results. Guinea pig coagulating gland. Aqueous or isotonic saline homogenates of guinea pig coagulating gland hydrolyze 100-300 μmoles TAMe per mg protein per hour. The bulk of the activity remained in the supernatant fluid after centrifugation at 3,500 x *g* for twenty minutes at 2°. The same preparations attacked benzoyl-L-arginine ethyl ester (BAEe) at about 50% of the rate of TAMe. However, benzoylglycine methyl ester, and the ethyl esters of the following amino acids were not hydrolyzed: L-leucine, L-cystine, N-phenylglycine, acetyl-DL-tryptophan, benzoyl-DL-α-alanine, L-lysine, DL-α-alanine and DL-tyrosine. At pH 7.5, guinea pig coagulating gland extracts barely hydrolyzed either casein or hemoglobin. The Michaelis constant for TAMe hydrolysis was estimated by the method of Lineweaver and Burk(7) from values for the rate of hydrolysis at different substrate concentrations, and was found to be 0.007 M. The hydrolysis of TAMe (0.03 M) was found to be unaffected by the following substances: ethylenediamine tetraacetic acid (6 x 10^{-4} M), $HgCl_2$ (4 x 10^{-5} M), $CuSO_4$ (9 x 10^{-5} M) $MnCl_2$ (4 x 10^{-4} M), $CaCl_2$ (2 x 10^{-3} M) and crystalline soy bean trypsin inhibitor (0.3 mg per ml). These concentrations of ethylenediamine tetraacetic acid and of $HgCl_2$ abolish the action of the coagulating enzyme vesiculase(1). The partial purification of the arginine ester hydrolyzing enzyme and its separation from vesiculase in guinea pig coagulating gland is shown in Table I.

TABLE I. Separation of Arginine Ester Hydrolyzing Enzyme from Vesiculase.

Fraction	Vesiculase activity	TAMe-splitting activity
(a) Ammonium sulfate fractionation of crude extract		
.3-.5 saturation	.81	210
.5-.8 "	0	1610
(b) Acetone fractionation of .3-.5 ammonium sulfate fraction		
0-20% acetone	2.44	21
20-30% "	1.93	23
30-50% "	.2	191

Crude extract was prepared by homogenization of the tissue in 0.9% NaCl, followed by centrifugation at 3,500 × *g* for 20 min. All operations carried out between 0° and 2°. All values expressed as units per mg protein. Vesiculase in arbitrary units. TAMe hydrolysis as μmoles split per hr.

TABLE II. Hydrolysis of TAMe by Rodent Accessory Sexual Tissues.

Organ	Guinea pig	Rat	Mouse	Hamster
Ventral prostate		14.8	.04	1.4
Dorsal "		.7	.06	.03
Lateral "	.7			.02
Coagulating gland	55.8	.02	.1	.02
Seminal vesicle	.3	.04	.2	.03

Tissues homogenized in 0.15 M KCl at 0°. Substrate concentration 0.03 M. Values represent μmole TAMe hydolyzed per mg wet weight of tissue per hour. Two experiments with mouse and hamster , in each of which tissue was pooled from 4 to 5 animals. Three experiments with guinea pig and 5 experiments with rat. The figures recorded are means of the values observed, which did not differ by more than 30%.

Distribution of arginine ester hydrolyzing activity in rodent accessory sexual tissues. The hydrolysis of TAMe by isotonic saline homogenates of the accessory reproductive organs of sexually mature male rodents is shown in Table II. In the guinea pig, only the coagulating gland hydrolyzes TAMe at significant rates. In the rat the coagulating gland exhibits negligible activity, but in this species TAMe is split rapidly by the ventral prostate gland. A similar situation is apparent in the hamster, whereas in the mouse none of the accessory sexual tissues examined hydrolyzed TAMe at more than negligible rates.

Intracellular distribution. Rat ventral prostate tissue was homogenized in 0.25 M sucrose-0.001 disodium ethylenediamine tetraacetate-0.003 M sodium bicarbonate, and various cellular components isolated by differential centrifugation according to Hogeboom, Schneider and Pallade(16). The fraction obtained after removal of nuclei and mitochondria was the only one which hydrolyzed TAMe at significant rates. The TAMe hydrolyzing activity of this fraction, which was not separated further into microsomal and "soluble" fractions, accounted for virtually all of the activity of the original homogenate.

Effect of castration. Table III shows that 8 days after castration the ability of rat ventral prostate homogenates to hydrolyze TAMe

TABLE III. Effect of Castration upon Hydrolysis of TAMe by Rat Ventral Prostate Gland.

Group	Wet wt of ventral prostate (mg)	μmole TAMe hydrolyzed/mg wet wt tissue/hr
Normal	333 (257-380)	13.9 (11.6-14.9)
Castrated 8 days	76 (65- 95)	5.9 (4.1- 8.4)

Substrate concentration 0.03 M. Figures in parentheses depict range of values observed. Five animals in each group.

was diminished to 46% of the normal level, whereas the wet weight of the glands had fallen to 23% of normal.

Canine and human prostatic fluids. Extremely rapid rates of TAMe hydrolysis by dog prostatic fluids were observed, 1 cc hydrolyzing from 10,000 to 30,000 μmoles per hour. These rates are equivalent to approximately 1,000 to 3,000 μmoles TAMe split per mg protein per hour. Most of the enzyme was recovered in the proteins precipitated by the addition of ammonium sulfate at 0° between 0.5 and 0.8 saturation. In contrast to the enzyme present in guinea pig coagulating gland and in the ventral prostate gland of the rat, canine prostatic fluid hydrolyzed BAEe at about twice the rate of TAMe. Dog prostatic fluids failed to hydrolyze the ethyl esters of either L-leucine or L-lysine. The arginine ester hydrolyzing enzyme of canine prostatic fluid was unaffected by heating to 60° for 10 minutes, but 90% of the activity was destroyed by heating to 70° for the same period of time. Centrifuged isotonic saline homogenates of whole prostatic glands removed within 3 hours post mortem from adult men without evidence of prostatic pathology hydrolyzed 0.2 to 0.4 μmole TAMe per mg protein per hour.

Discussion. The arginine ester hydrolyzing enzyme of rodent and canine prostatic fluid bears a strong resemblance to the human seminal enzyme which hydrolyzes BAEe described by Lundquist *et al.*(8). Thus both enzymes are unaffected by ethylenediamine tetraacetic acid, and appear to be quite distinct from trypsin-like enzymes which degrade casein. Although both TAMe and BAEe are substrates for trypsin(9), the latter enzyme also attacks L-lysine ethyl ester, which is not hydrolyzed by the prostatic enzyme. The lack of identity of the prostatic enzyme with trypsin is further established by the failure of crystalline soy bean trypsin inhibitor to inhibit its activity. Sherry and his coworkers(10,11) have shown that both thrombin and plasmin will hydrolyze TAMe. The latter enzyme also hydrolyzes L-lysine ethyl ester, and would thus appear to be different from the prostatic enzyme described here. Moreover, canine prostatic fluid, which is practically devoid of fibrinolysin(12), splits TAMe at extremely rapid rates, whereas human prostatic fluid, which is rich in fibrinolysin(12,13) hydrolyzes TAMe rather slowly. Canine prostatic fluids have little ability to clot fibrinogen(12), which suggests that the prostatic arginine ester hydrolyzing enzyme is not identical with thrombin. Moreover, crude extracts of the guinea pig coagulating gland, which hydrolyze TAMe rapidly, will not clot fibrinogen(1). Lundquist *et al.*(8) concluded that the human seminal enzyme which splits BAEe is not identical with trypsin, thrombin or seminal fibrinolysin, and a similar conclusion can be reached concerning the enzyme investigated here, which is probably the same as that described by Lundquist.

The ready separation of vesiculase from the arginine ester hydrolyzing enzyme in the guinea pig coagulating gland shows that these two enzymes are not identical. The distribution of these two enzymes in the accessory reproductive organs of other rodents also negates the possibility that the arginine ester hydrolyzing enzyme is primarily concerned with the coagulation process. Thus in the rat and in the hamster the ventral prostate gland is the only accessory sexual tissue which splits TAMe, while vesiculase activity is confined to the coagulating gland(1,14). It has been pointed out elsewhere(1) that TAMe itself, which inhibits the clotting activity of the thrombin(11), does not affect vesiculase activity.

The extremely rapid rates of TAMe hydrolysis by only some lobes of the prostate gland of a given species, and the remarkable differences in the distribution of this arginine

ester hydrolyzing enzyme among the accessory reproductive organs of various animals is reminiscent of similar tissue and species differences in the manufacture of other components of the seminal plasma such as fructose, citric acid, inositol and acid phosphatase (15). The immense rates of arginine ester hydrolysis by some accessory sexual tissues, and the fall in activity induced by castration, adds this arginine ester hydrolyzing enzyme to the already imposing list of chemical accessory sexual characteristics in the male.

Summary. Certain esters of arginine, but not those of a number of other amino acids, are hydrolyzed rapidly by some lobes of the prostate gland of a number of species. Marked differences in the activity of this arginine ester hydrolyzing enzyme in different lobes of the prostate gland of various rodents have been observed. Dog prostatic fluid is extremely rich in this enzyme, whereas the human prostate hydrolyzes arginine esters rather slowly. The activity of the enzyme in rodent prostatic tissue is depressed after castration. Some properties of this enzyme, and its relationship to other proteolytic enzymes in prostatic secretion as well as to the process of semen coagulation are discussed.

1. Gotterer, G., Ginsburg, D., Schulman, T., Banks, J., and Williams-Ashman, H. G., *Nature,* 1955, v176, 1209.

2. Werbin, H., and Palm, A., *J. Am. Chem. Soc.,* 1951, v73, 1382.

3. Warburg, O., and Christian, W., *Biochem. Z.,* 1941, v310, 384.

4. Kunitz, M., *J. Gen. Physiol.,* 1947, v30, 291.

5. Anson, M. M., *ibid.,* 1938, v22, 79.

6. Huggins, C., Masina, M. H., Eichelberger, L., and Wharton, J. D., *J. Exp. Med.,* 1939, v70, 543.

7. Lineweaver, H., and Burk, D., *J. Am. Chem. Soc.,* 1934, v56, 658.

8. Lundquist, F., Thorsteinsson, Th., and Buus, O., *Biochem. J.,* 1955, v59, 69.

9. Neurath, H., and Schwert, G. W., *Chem. Rev.,* 1950, v46, 69.

10. Troll, W., Sherry, S., and Wachman, J., *J. Biol. Chem.,* 1954, v208, 85.

11. Sherry, S., and Troll, W., *ibid.,* 1955, v208, 95.

12. Huggins, C., and Neal, W., *J. Exp. Med.,* 1942, v76, 527.

13. Huggins, C., *The Harvey Lectures,* 1947, v42, 148.

14. Walker, G., *Bull. Johns Hopkins Hosp.,* 1910, v21, 182.

15. Mann, T., and Lutwak-Mann, C., *Physiol. Revs.,* 1951, v31, 27.

16. Hogeboom, G. H., Schneider, W. C., and Pallade, G. E., *J. Biol. Chem.,* 1948, v172, 619.

Received March 5, 1956. P.S.E.B.M., 1956, v92.

Influence on Hepatic Ferritin Systems of Tertiary Amine, G-D 131, with Beneficial Effects in Shock.* (22385)

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This report deals with effects and mode of action, in experimental shock, of a group of synthetic agents not previously explored, since they possessed no known properties suggestive of potential value as protective agents in shock. They are tertiary amines, chemically related to β-chloroalkylamines, adrenergic blocking agents, of which N, N-dibenzyl-β-chloroethylamine (Dibenamine) is the prototype. These compounds are structurally altered so that their adrenergic blocking properties have been lost. Several of these compounds were utilized by Nickerson and Gump (1) in their study of the relation between chemical structure and adrenergic blocking activity; others have been synthesized for the current study. This group of compounds was

* Aided by research grants from Josiah Macy, Jr. Foundation, N. I. H., U. S. Public Health Service (Grant H-79), Armour Laboratories, Department of Army and the Postley Fund.

† Deceased Jan. 6, 1956.

selected on the basis of our analysis of mechanisms by which related adrenergic blocking agents provide high degree of protection in experimental shock which we(2) and other workers(3,4) have described. We observed two distinct actions of Dibenzyline: one, resulting from adrenergic blockade, was the blunting of the usual extreme peripheral vasoconstrictive response to hemorrhage or trauma, which affords a better perfusion of vital splanchnic organs throughout the shock syndrome(5); the other was a specific effect on the ferritin (VDM) regulating mechanisms of the liver(6). At low dosage levels *in vivo*, and by brief exposure to it *in vitro*, Dibenzyline inhibits normal anaerobic formation of vasoactive ferritin and also preserves the aerobic ferritin-inactivating system from deterioration ordinarily resulting from prolonged hepatic hypoxia. These observations bear on the concept of shock advanced by us (7), which would assign a major role in determining the outcome to the liver and, specifically, to the hepatic systems regulating metabolism of ferritin. These systems are believed to contribute to irreversibility of shock syndrome when they deteriorate to such a degree, from prolonged hypoxia, that the liver loses its capacity to inactivate the hepatic vasodepressor, ferritin, aerobically and despite restoration of aerobic conditions by transfusion, continues to elaborate this vasoactive agent, with its potentially unfavorable influence on the dynamics of the splanchnic terminal vascular bed. With Dibenzyline, our observations were compatible with this concept; but a more direct test of this hypothesis would be provided by agents which act only on hepatic ferritin systems, in the same manner as Dibenzyline, but without its additional adrenergic blocking action. It seemed reasonable to look for such compounds in the group, described above, which preserved certain chemical features of active adrenergic blocking agents but had lost this pharmacologic property.

The present report concerns the actions of N - (2-chloroethyl) - N-(cyclohexylmethyl)-ethylamine hydrochloride (G-D 131), whose loss of adrenergic blocking action was attributed by Nickerson and Gump(1) to saturation of the benzene ring, the unsaturated homologue being highly active.

Methods. Female rats of Wistar strain, weighing 120 to 150 g, were used. For *in vitro* studies, rats were killed by a blow on the head, liver removed immediately and sliced as for microrespiratory studies. The slices were placed in Erlenmeyer flasks containing Ringer-phosphate medium, pH 7.4, 5 volumes of medium to one gram of tissue. G-D 131 was added 2 μg/ml (10 μg/g liver) before incubating at 37.5° C in oxygen for 10 minutes. Slices from the same liver, treated similarly except for omission of the drug, served as controls. After incubation the media were discarded and the slices washed twice with Ringer-phosphate. For studies of anaerobic VDM formation, fresh Ringer-phosphate, in the same (1:5) proportion was added to the slices which were then incubated in N_2 for 90 minutes. Then the clear supernatant liquid was bioassayed by the rat mesoappendix method(8). The same slices were then reincubated, to test their ferritin-inactivation capacity, in O_2 in Ringer-phosphate containing known amount of vasoactive ferritin (usually 0.001 μg ferritin N/ml). After 2 hours the media were bioassayed as above. *Traumatic shock* experiments were carried out by the method of Noble and Collip(9) in unanesthetized rats, using 715 rotations of the drum at 44 r.p.m. 200 μg G-D 131‡/100 g body wt were given intravenously 60 or 180 minutes before drumming. A parallel set of saline-injected controls was drummed the same day. *Hemorrhagic shock* was induced under light anesthesia by graded bleeding, controlled by self-infusion apparatus(10). G-D 131 (80 μg/100 g body wt in 0.2 ml saline) was given intravenously one hour before start of experiment. Controls received 0.2 ml saline, while another series were given 20 μg Dibenzyline/100 g body wt i.v. at the same time interval. Heparin was present in saline and blood reservoir system in all experiments. Blood pressure was maintained at

‡ Kindly supplied by Dr. Mark Nickerson, Univ. of Manitoba and Dr. William Gump, Givaudan-Delawanna Corp.

or above 70 mm Hg for the first hour, at 50 mm Hg during the following hour and 30 mm Hg for remaining 2 hours. At end of the 4 hour period blood remaining in reservoir was slowly force-infused. Direct microscopic observation of terminal vascular bed in the mesoappendix was carried out throughout the hemorrhagic procedure in a number of animals(8). Ability to restrict VDM release to anaerobiosis and the capacity to inactivate ferritin aerobically were examined in livers removed from a number of rats at the end of the 4 hour hypotensive period. For this purpose liver slices were incubated aerobically in Ringer-phosphate for one hour, or in Ringer-phosphate containing vasoactive ferritin for 2 hours. Media were then bioassayed by rat test.

Results. Properties of G-D 131. Absence of adrenergic blocking properties, reported by Nickerson and Gump(1), was confirmed in the rat for amounts up to 200 μg/100 g body wt, the maximal amount administered in present experiments. At this dosage level, it was also without anticholinergic action.

Dibenzyline, in amounts which provide excellent protection in shock (*i.e.* 5 μg or more /100 g body wt), depresses reactivity to topical epinephrine of muscular capillaries of the normal rat; whereas G-D 131, at dosage levels up to 200 μg/100 g body wt was without effect on epinephrine reactivity, vasomotion or any other characteristic of blood flow in the mesoappendix of rats examined immediately, or up to 3 hours, after administration of drug. G-D 131 was tested for antibiotic activity§ and had none in concentrations of 50 and 250 μg against 8 organisms (*Staphylococcus aureus, Streptococcus* (Group A)-*C-203, Proteus vulgaris, Escherichia coli, Salmonella, Klebsiella pneumoniae, Clostridium welchii, Pseudomonas aeruginosa*). In the method employed Aureomycin is effective for all these organisms at concentration of 50 μg and for *Cl. welchii* at 3 μg.

Protection of hepatic ferritin mechanisms against anaerobic deterioration by brief exposure to G-D 131 in vitro. Our studies of *in vitro* action of Dibenzyline(6) on normal liver ferritin mechanisms suggested that similar experiments might be used to select, from this large series of tertiary amines, those most likely to be of value in shock experiments. Table I summarizes the results obtained with G-D 131 in *in vitro* studies of livers removed from normal rats. In agreement with our earlier results control liver slices released vasoactive ferritin into the medium during anaerobic incubation(11); under similar conditions, those briefly exposed to G-D 131 did not do so. Control liver slices, exposed to anaerobiosis, uniformly failed to inactivate added ferritin on subsequent aerobic incubation, while those briefly exposed, before anaerobiosis, to G-D 131 retained their normal ferritin-inactivating capacity. Thus, despite chemical changes which deprive it of adrenergic blocking activity, G-D 131 resembles Dibenzyline in its specific influence on ferritin regulating mechanisms of liver, and it therefore seemed an appropriate agent for evaluating the relation of these ferritin mechanisms to recovery from shock.

TABLE I. *In Vitro* Effect of G-D 131* on the Hepatic Ferritin Systems.

VDM Activity	Treatment of liver slices after exposure to G-D 131	
	N_2 x 90 minutes	N_2 x 90 minutes, then O_2 + ferritin x 120 minutes
Strong	● ● ● ●	● ● ● ● ● ● ● ●
Moderate	● ● ● ●	● ● ●
Mild	● ●	○
Neutral	○ ○ ○ ○ ○ ○ ○ ○ ○ ○	○ ○ ○ ○ ○ ○ ○ ○ ○ ○

○ = G-D 131 ● = Controls

* Liver slices (1 g) incubated 10 min. at 37.5°C with G-D 131 (10 μg) in 5 ml Ringer PO_4 medium in 100% O_2. Slices washed 2 times and reincubated as above.

G-D 131 in traumatic shock. The percent survivals of controls and rats treated with G-D 131 at 60 or 180 minutes before rotation in the drum, are given in Table II. Although the small difference between controls and

§ By Drs. R. M. McCune, Jr. and P. Dineen at Cornell University Medical College.

TABLE II. Effect of Intravenous Pretreatment with G-D 131 on the Outcome of Drum Trauma in the Rat.

Treatment	Saline	200 μg G-D 131/100 g body wt	
Time of Inj.	60 to −180 min.	−60 min.	−180 min.
No. of rats	94	28	93
" " rotations	715	715	715
" surviving at 24 hr	41	15	78
% survival at 24 hr	44	53	84

those treated 60 minutes before shock is not significant, when G-D 131 is given 3 hours before drumming the survival is almost doubled, 84% as compared with 44% in controls.

G-D 131 in hemorrhagic shock. Data obtained on effects of pretreatment with Dibenzyline or G-D 131 on outcome of hemorrhagic shock are presented in Table III. The shock was profound and resulted in survival of only 27% in controls. The recovery rate with G-D 131 was 77%, and 63% with Dibenzyline.

During the first few hours of the hemorrhagic experiment, direct observations of blood flow in mesoappendiceal vessels disclose a close resemblance between controls and G-D 131 treated rats. However, as hypotension is prolonged, in control animals there is progressive deterioration of circulation, with decline in reactivity to topical epinephrine, loss of vasomotion and eventual stagnation of blood in the capillary bed. In treated animals a compensatory type of peripheral vascular behavior persists throughout the experiment, with preservation of enhanced reactivity to epinephrine, and vasomotion, and with good flow throughout the capillary bed. In Dibenzyline-treated rats the originally depressed reactivity to topical epinephrine is rapidly replaced by compensatory type of behavior persisting throughout the syndrome.

TABLE III. Comparison of the Effect of Dibenzyline and G-D 131 on the Outcome of Hemorrhagic Shock in the Rat.*

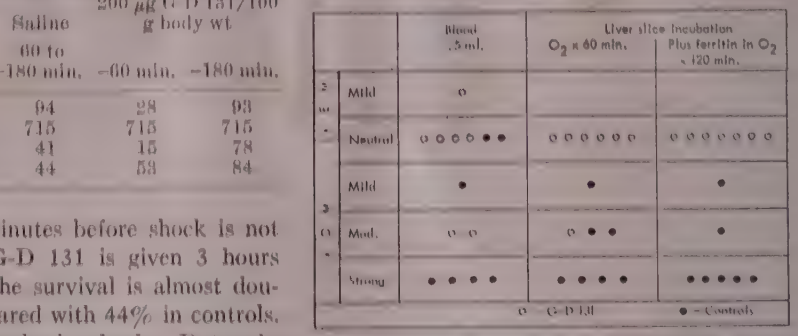
Inj. into tail vein	Controls, .2 ml saline	Dibenzyline, 20 μg/100 g body wt	G-D 131, 80 μg/100 g body wt
No. of animals	22	19	22
Max blood lost† (% body wt)			
Avg	4.5	4.5	4.1
Range	3.5–5.3	3.3–5.5	3.5–5.0
Uptake of blood‡ (% body wt)			
Avg	1.0	.5	.5
Range	.0–3.6	.1–1.0	.0–1.8
% survival at 24 hr	27	63	77

* Both agents Inj. 60 min. before bleeding.

† Total blood loss required to bring blood pressure to 30 mm Hg level.

‡ Amount of blood spontaneously taken up from reservoir to maintain blood pressure at 30 mm Hg for 2 hr.

TABLE IV. Effect of Pretreatment with G-D 131* on Hepatorenal Vasoactive Factors in Blood and Liver after Four Hours of Hemorrhagic Hypotension.†



* G-D 131 80 μg/100 g body wt was given into tail vein 60 min. before hemorrhagic procedure.

† At or above 70 mm Hg for first hr, at 50 mm Hg for second hr, and at 30 mm Hg for following 2 hours.

The status of the hepatic ferritin regulating systems at end of the standard 4 hour hemorrhagic hypotensive period was investigated in another series of rats (Table IV). In contrast to liver slices from control shocked rats, which now released vasoactive ferritin in oxygen and had completely lost their aerobic ferritin-inactivating capacity, livers from G-D 131 treated animals behaved as do normal unshocked livers, releasing no ferritin aerobically and inactivating added vasoactive ferritin. Bioassays of blood samples drawn at end of the hemorrhagic experiment were generally confirmatory of findings in livers; blood from treated animals usually contained no VDM while the opposite was true of controls. From these results it is apparent that protection afforded by G-D 131, against deterioration of the ferritin-inactivating systems during *in vitro* hepatic hypoxia, is also exerted in the living animal during hemorrhagic hypotension.

Discussion. A tertiary amine, N-(2-chloro-

ethyl)-N-(cyclohexylmethyl)-ethylamine hydrochloride (G-D 131), related structurally to Dibenamine type of adrenergic blocking agent, but altered so that adrenergic blocking properties are lost, shares with Dibenzyline the property of modifying the ferritin regulating mechanisms of the liver. *In vitro*, brief exposure to 10 μg/g of tissue results in inhibition of the usual anaerobic release of vasoactive ferritin (VDM) from normal liver slices and in preservation of the aerobic hepatic ferritin-inactivation mechanism despite prolonged anaerobiosis. *In vivo*, pretreatment of rats with 200 μg of G-D 131/100 g body wt almost doubles survival rate in drum shock, and administration of 80 μg/100 g (approximately 1/100 of the LD_{50}) results in protection against hemorrhagic shock equal to that provided by Dibenzyline which had hitherto proved to be the most effective protective agent. Liver ferritin mechanisms, examined in G-D 131 treated rats following hemorrhagic procedure, compare favorably with those of normal unshocked rats, releasing no ferritin aerobically and retaining normal ferritin-inactivation capacity, in contrast to livers of control rats which now release ferritin aerobically and are unable to inactivate added ferritin.

Since G-D 131 is devoid of observable direct effects on behavior of terminal vascular bed of mesoappendix of the normal rat, maintenance of compensatory peripheral vascular activity throughout hemorrhagic shock procedure would not appear to be attributable to a direct influence of the drug, but may be a consequence of its action in preventing release into the blood stream of deleterious factors from the liver.

The failure of G-D 131 to protect against drum shock unless injected 3 hours before trauma (see Table II) appears paradoxical in view of our previous finding(12) that it exerts a protective effect in hemorrhagic shock when administered as late as 90 minutes after start of procedure. However, one of the differences between the two shock procedures may be related to this time factor; the drastic hemorrhagic hypotension may be assumed to become maximally harmful only after it has been maintained for several hours while in drum trauma the entire stress is imposed within the 15 minute period of rotation in the drum. The possibility is being investigated that a lapse of some time is required for G-D 131 to exert its maximum effect on the hepatic ferritin systems *in vivo*. A comparable time requirement has been noted with Aureomycin in drum shock(13)—the protection exerted is greater when this agent is injected 2 hours rather than one hour before trauma.

Summary. These observations with G-D 131 are regarded as support for our concept that deterioration of the hepatic ferritin mechanisms brings about consequences of major importance for ability of the animal to survive a standardized shock procedure.

1. Nickerson, M., and Gump, W. S., *J. Pharmacol. and Exp. Therap.*, 1949, v97, 25.

2. Baez, S., Zweifach, B. W., and Shorr, E., *Fed. Proc.*, 1952, v11, 7.

3. Wiggers, H. C., Ingraham, R. C., Roemhild, F., and Goldberg, H., *Am. J. Physiol.*, 1948, v153, 511.

4. Remington, J. W., Hamilton, W. F., Boyd, G. H., Jr., Hamilton, W. F., Jr., and Caddell, H. M., *ibid.*, 1950, v161, 116.

5. Zweifach, B. W., Baez, S., and Shorr, E., *Fed. Proc.*, 1952, v11, 177.

6. Baez, S., Srikantia, S. G., Forbes, I., and Shorr, E., *ibid.*, 1956, v15, 390.

7. Shorr, E., Zweifach, B. W., Furchgott, R. F., and Baez, S., *Circulation*, 1951, v3, 42.

8. Zweifach, B. W., *In Methods in Medical Research* (V. R. Potter, ed.), 1948, v1, 131, Year Book Publishers, Chicago.

9. Noble, R. L., and Collip, J. B., *Quart. J. Exp. Physiol.*, 1942, v31, 187.

10. Baez, S., Zweifach, B. W., Pellon, R., and Shorr, E., *Am. J. Physiol.*, 1951, v166, 658.

11. Shorr, E., Zweifach, B. W., and Furchgott, R. F., *Ann. New York Acad. Sc.*, 1948, v49, 571.

12. Srikantia, S. G., Baez, S., Carleton, A., and Shorr, E., *Fed. Proc.*, 1956, v15, 177.

13. Baez, S., and Shorr, E., *Shock and Circulatory Homeostasis* (H. D. Green, ed.), Tr. Fifth Conf., Josiah Macy, Jr. Foundation, New York, 1955, in press.

Received March 5, 1956. P.S.E.B.M., 1956, v92.

Anaerobic Studies of Steroidogenesis Using Perfused Calf Adrenals. (22386)

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In earlier studies with isolated calf adrenals(1), it was observed that suitable conditions for the biogenesis of corticoids were afforded if the glands were perfused at body temperature with a well-oxygenated artificial medium. However, when oxygenation was omitted, negligible production of adrenocortical steroids occurred. In subsequent studies of adrenal intermediary metabolism in which 2, 3, 5-triphenyl tetrazolium chloride (TTC) was employed in place of oxygen as a hydrogen acceptor(2), no corticoids were produced despite concurrent oxidation of various Meyerhof-Embden or Krebs cycle intermediates added to the perfusion medium. Rapid utilization of these substrates was manifested by appreciably larger deposition of formazan (reduced TTC) in the substrate-exposed adrenals than in contralateral control organs. These findings suggested that "molecular oxygen or a reactive derivative, *e.g.*, $^{\bullet}OH$, $HO_2^{\bullet}$, H_2O_2, as opposed to hydroxyl ions derived from water, was essential for formation of one or more of the corticosteroid oxygen functions(2)." Recently, Hayano and coworkers(3), employing H_2O^{18}, demonstrated that molecular oxygen, but not water, was utilized by the adrenal 11β-hydroxylase system in C-11β-hydroxylation of steroids. It will be recalled that in the steroidogenic reaction sequence, this biomodification is usually preceded by 17α-and/or 21-hydroxylation(4,5). Accordingly, if the failure of actively metabolizing glands to elaborate compounds F and B† were attributable solely to an inability to perform 11β-hydroxylation under anaerobic conditions, one might anticipate an accumulation of 11-desoxy precursors, *viz.*, Compound S and DOC. Absence of these or other steroidal intermediates suggested that among the large variety of enzymes participating in formation of corticoids from acetate or cholesterol, there may be other systems which require molecular oxygen. The main objective of the present investigation was to test this hypothesis by perfusing calf adrenals anaerobically with the artificial medium containing TTC and either (a) ACTH or (b) model steroids as substrates.

Materials and methods. Weighed calf adrenals, varying from 2.5 to 5.0 g, were excised at a local slaughterhouse, and dissected free of periadrenal fat and connective tissue. They were kept in an ice-cold, citrate-saline solution until cannulated and perfused via the adrenal vein. Details of the perfusion technic have already been presented(1). Control groups of 2 left and 2 right glands were perfused at 37.5°C in a multicycle system with a liter of artificial medium, continuously treated with a stream of 95% O_2/5% CO_2 and composed of the following substances, in grams per liter: glucose 2.00, NaCl 8.2, KCl 0.20, $CaCl_2$ 0.20, $MgSO_4$ 0.13, Na HCO_3 0.60, Na H_2PO_4 0.05. Experimental groups, comprising the 4 contralateral organs and differing in weight by less than 4%, were simultaneously perfused in another apparatus with a liter of the same medium. However, the latter was continuously treated with a stream of nitrogen and contained 2.5 grams of dissolved 2, 3, 5-triphenyl tetrazolium chloride (TTC). Use of this non-toxic, water-soluble tetrazolium salt as a physiological tool depends upon its interaction as a hydrogen acceptor with intracellular reductases, such as dehydrogenases, and its subsequent enzymatic reduction to an insoluble formazan. It is probable that TTC ($E'_o = -0.08$ volt) is reduced by a variety of dehydrogenase systems requiring

* The opinions or assertions contained herein are the private ones of the writer and are not to be construed as official or reflecting the views of the Navy Department or the naval service at large.

† These compounds and cortisone (E) are the major components of the steroidal secretion of the perfused calf adrenal(1).

TABLE I. Corticoid Output of Contralateral Groups of ACTH-Stimulated* Calf Adrenals Perfused *In Vitro* with an Oxygenated or TTC-Fortified† Artificial Medium.

Treatment of perfusion medium	μg/hr/4 glands			
	Cortisol (F)	Cortisone (E)	Corticosterone (B)	F, E & B
O_2	858	217	304	1379
N_2 + TTC	0	0	26	26
O_2	571	216	545	1332
N_2 + TTC	0	0	51	51
O_2‡	925	156	259	1340
N_2 + TTC‡	30	8	0	38

* 25 I.U. of ACTH (Armour's *ACTHAR*).
† 2.5 g/l of 2, 3, 5-triphenyl tetrazolium chloride.
‡ Mean values for 2 perfusion experiments in which the control (O_2) and experimental (TTC) effluents were combined before analysis.

coenzymes I or II(6) and that flavoproteins are the immediate electron donors(7). Previous studies(2) have shown that TTC competes with oxygen as a hydrogen acceptor, and that its reduction by the perfused calf adrenal to the stable, water-insoluble, deep-red pigment is essentially enzymatic. Either ACTH (25 I. U. of Armour's ACTHAR) or one of the diverse steroid substrates employed, *viz.*, Compound S, DOC, 21-desoxycortisone, progesterone and dehydroisoandrosterone dissolved in propylene glycol, was added to the circulating fluids of both the experimental and control glands. After a 1-hour perfusion, the effluents were collected and extracted with ethyl acetate. The steroid residues were fractionated by paper partition chromatography, and eluates of the various zones assayed by the Porter-Silber (Compounds F and E), phosphomolybdic acid (Compound B), and Zimmermann (C_{19}-ketosteroids) reactions. The extraction, chromatography, and assay procedures have been described in detail(8,9).

Results. Table I embodies analytical data of 4 experiments in which contralateral groups of ACTH-stimulated calf adrenals were concurrently perfused for 1 hour with the oxygenated or TTC-fortified artificial medium. It clearly demonstrates that glands perfused anaerobically (N_2 plus TTC) secreted little or no Compounds F, E and B in marked contrast to control organs perfused aerobically (95% O_2/5% CO_2). That this disparity in output was due to failure in biosynthesis rather than release of corticoids was shown by analysis of the glands following perfusion. The experimental organs contained amounts of corticoids comparable to those recovered from unperfused glands and totaling about 25% of the residual content of the perfused contralaterals (approx. 110 μg/4 glands).

It must be emphasized that under the experimental conditions TTC does not appreciably destroy, alter or combine with Compounds F, E or B nor does it interfere with their chromatographic mobility, identification, or measurement. Recoveries of 94, 93 and 107% were observed after a liter of artificial medium, containing 2.5 g TTC and a mixture of these steroids (510, 210 and 310 μg, respectively) had been circulated for one hour in a perfusion apparatus without mounted organs.

Table II summarizes the results of 5 conversion studies in which contralateral groups of unstimulated calf adrenals were concurrently perfused for one hour with the oxygenated or TTC-fortified artificial medium containing an added steroid substrate. The major transformation products were isolated and determined as previously described; their identification by infrared analysis and melting point has already been reported(8). Although the aerobically perfused glands 11β-hydroxylated substantial quantities of 11-desoxycortisol and 11-desoxycorticosterone to cortisol and corticosterone, respectively, anaerobically perfused glands exhibited negligible 11β-hydroxylase activity. A similar dependence of the 21-hydroxylating mechanism on the presence of molecular oxygen is evidenced by the 21-desoxycortisone → cortisone conversion study. With respect to 17α-hydroxylation, appreciable amounts of Compound F (17-OH-corticosterone) were recovered following aerobic but not anaerobic perfusion of progesterone. Although Compounds F and B are the quantitatively major end-products, small amounts of 17α- and 11β-hydroxyprogesterone have also been reported (10). Control (O_2) chromatograms did reveal a moderate quantity of the latter inter-

TABLE II. Conversion of Steroid Substrates by Contralateral Groups of Calf Adrenals Perfused *In Vitro* with an Oxygenated or TTC-Fortified Artificial Medium.

Steroid substrate and major transformation product(s)	Amt of substrate perfused (mg)	Major product(s) formed in 1 hr (mg)		Specific enzymatic reaction(s)
		O_2	TTC(N_2)	
11-Desoxycortisol (S)	20			
↓				11β-OH
Cortisol (F)		15.5	.3	
11-Desoxycorticosterone	30			
↓				11β-OH
Corticosterone (B)		13.4	.4	
21-Desoxycortisone	20			
↓				21-OH
Cortisone (E)		10.8	.6	
Progesterone	40			
↓				17α-, 21-, 11β-OH
Cortisol (F)		2.5	0	21-, 11β-OH
↘				
Corticosterone (B)		3.9	0	
Dehydroisoandrosterone	25			
↓				Δ^5-3β-OH →
Δ^4-androstene-3,17-dione		1.6	7.5	Δ^4-3-ketone
↓				11β-OH
11β-OH-Δ^4-androstene-3,17-dione		4.4	.1	

mediates but there were no corresponding zones on the experimental ones.

In marked contrast to the preceding enzymatic oxidations, the conversion of the Δ^5-3β-hydroxyl group to the Δ^4-3-ketone readily occurred in the absence of molecular oxygen. Table II demonstrates that the anaerobically perfused organs produced a total of 7.6 mg of Δ^4-androstene-3, 17-dione compounds as compared with the control value of 6.0 mg. Inability to 11β-hydroxylate Δ^4-androstene-3, 17-dione formed corroborates the previous finding that this biosynthetic reaction is oxygen-dependent.

To ascertain whether anaerobically perfused, actively metabolizing adrenals produced appreciable quantities of a C_{19} or C_{21} precursor or intermediate of the adrenocortical hormones, the combined steroid residues of 2 ACTH-stimulation experiments were subjected to a series of successive paper chromatographic fractionations in toluene-propylene glycol(11) and ligroin-propylene glycol partition systems(12). In view of the demonstrated 3β-dehydrogenase activity of the glands under anaerobic conditions, it was reasoned that there might be an accumulation of progesterone (and Δ^4-androstene-3, 17-dione)‡ if oxygen were not needed in the biosynthetic reactions preceding formation of Δ^5-pregnenolone (and dehydroisoandrosterone, the putatively important intermediates in costeroidogenesis). A 72-hour partition in the toluene system followed by an 18-hour resolution of the runoff revealed a weakly positive cortisol zone but no other zones absorbing in the UV or positive to triphenyltetrazolium chloride (TPTZ) or Zimmermann color tests. Thus, no significant amounts of cortisone (E), 11-desoxycortisol (S) or corticosterone (B) were detected. The 18-hour toluene overflow was systematically chromatographed for 48, 24 and 8 hours in the ligroin system. Essentially negative UV scanning and spot tests of these paper chromatograms precluded the possibility of significant amounts of 11β- or 17α-hydroxyprogesterone, 11-desoxycorticosterone (DOC), dehydroisoandrosterone, Δ^4-androstene-3, 17-dione, Δ^5-pregnenolone or progesterone. Pure samples of all the aforementioned steroids were concurrently chromatographed on 1-cm

‡ We have observed at most only minute amounts of C_{19} steroids in the effluent of the isolated perfused calf adrenal.

strips as reference standards and identified. Absence of a substantial quantity of one or more $C_{21}O_2$ steroids in the experimental effluents stands in sharp contrast to the control effluent content which comprised, aside from other quantitatively minor components, a total of approximately 3 mg of Compounds F, E and B. Thus, it would appear that molecular oxygen is required not only in the 17α-, 21-, and 11β-hydroxylation systems but also in one or more of the preliminary enzymatic reactions which result in formation of the $C_{21}O_2$ steroid nucleus.

Discussion. It is noteworthy that the 3β-dehydrogenase system, which does not require an oxygen atmosphere, catalyzes an oxidation reaction entailing a net loss of hydrogen, whereas the 3 oxygen-dependent hydroxylating systems examined catalyze an oxidation resulting in a net increase in the oxygen content of the steroidal end-product. The latter findings recall the observations made by Conn *et al.*(13) of an enzyme system in wheat germ involving a peroxidase which catalyzes the oxidation of TPNH by molecular oxygen, and by Brodie *et al.*(14) of a peroxidase-like system in liver microsomes which requires TPNH and oxygen to hydroxylate aromatic compounds. It is possible that there may be similar peroxidase-like systems in adrenocortical tissue which with TPNH and molecular oxygen selectively introduce hydroxyl groups into the steroid nucleus, perhaps via peroxide formation. This hypothesis appears more plausible if one recalls that the 11β-, 17α-, and 21-hydroxylase mechanisms have all been shown to require TPN or TPNH for optimal activity(4).

In evaluating the present data vis-a-vis the dependence of these hydroxylases on molecular oxygen, it is important to consider the quantitative aspects and nature of the cellular energy produced anaerobically in relation to the oxidative changes studied. Under the experimental conditions, $\Delta F \cong -6000$ cals/mole TTC reduced. Moreover, since oxidation of a methyl group or ring methylene group to the corresponding methylol or secondary hydroxyl function involves a liberation of free energy, it is reasonable to assume that oxidation of C-21 or C-11 would likewise have a negative ΔF and entail liberation of free energy whether coupled to O_2 or TTC as the final electron acceptor. Although it is difficult to estimate the free energy change involved in the C-17α oxidation without an appropriate model for comparison, it seems reasonable that, if not exergonic, at least it does not require an unavailable level of free energy input.

With respect to the intermediary metabolism of the TTC-perfused adrenals, previous studies(2) have demonstrated that all of the added Meyerhof-Embden and Krebs-Johnson carbohydrate intermediates were readily utilized. Thus, whether the energy is derived from the concurrent functioning of the glycolytic and/or the terminal aerobic pathway (15,16), it would appear from the aforementioned considerations of the free energy and oxidation changes that active reduction of TTC would support the steroid hydroxylating mechanisms if they proceeded independently of molecular oxygen.

Summary. Isolated, ACTH-stimulated calf adrenals, perfused anaerobically (N_2) with TTC as a hydrogen acceptor, failed to elaborate Compounds F, E, B or a $C_{21}O_2$ steroidal intermediate despite an active oxidative metabolism. Specific enzymatic conversion studies with added steroid substrates demonstrated their inability to perform 11β-, 17α-, and 21-hydroxylations under anaerobic conditions although they were able to transform the Δ^5-3β-hydroxyl group to the Δ^4-3-ketone. It would appear that the hydroxylases, which selectively introduce hydroxyl groups in the side chain or nucleus, require molecular oxygen (or a reactive derivative) in contrast to the 3β-dehydrogenase system, which catalyzes the oxidation of an existing oxygen function.

The authors are grateful to H. B. Bond for his valuable technical assistance.

1. Rosenfeld, G., *Endocrinology*, 1955, v56, 649.

2. ———, to be published in *Arch. Biochem. Biophys.*

3. Hayano, M., Lindberg, M. C., Dorfman, R. I., Hancock, J. E. H., and Doering, W. von E., *ibid*, 1955, v59, 1.

4. Hechter, O., and Pincus, G., *Physiol. Rev.,* 1954, v34, 459.

5. Dorfman, R. I., *The Hormones,* Academic Press Inc., 1955, v3, 589.

6. Mattson, A. M., Jensen, C. O., and Dutscher, R. A., *Science,* 1947, v106, 294.

7. Straub, F. B., *Biochem. J.,* 1939, v33, 787.

8. Rosenfeld, G., Ungar, F., Dorfman, R. I., and Pincus, G., *Endocrinology,* 1955, v56, 24.

9. Rosenfeld, G., and Ungar, F., *ibid.,* 1955, v56, 30.

10. Levy, H., Jeanloz, R. W., Jacobsen, R. P., Hechter, O., Schenker, V., and Pincus, G., *J. Biol. Chem.,* 1954, v211, 867.

11. Zaffaroni, A., Burton, R. B., and Keutmann, E. H., *Science,* 1950, v111, 6.

12. Savard, K., *J. Biol. Chem.,* 1953, v202, 457.

13. Conn, E. E., Kraemer, L. M., Liu, P., and Vennesland, B., *ibid.,* 1952, v194, 143.

14. Brodie, B. B., Axelrod, J., Cooper, J. R., Gaudette, L., La Du, B. N., Mitoma, C., and Udenfriend, S., *Science,* 1955, v121, 603.

15. Hayano, M., and Dorfman, R. I., *J. Biol. Chem.,* 1953, v201, 175.

16. Brownie, A. C., and Grant, J. K., *Biochem. J.,* 1954, v57, 255.

Received March 5, 1956. P.S.E.B.M., 1956, v92.

Effect of Eastern Equine Encephalomyelitis Virus Infection on Phosphate Transfer and Modification by Cortisone.* (22387)

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The biochemical approach to the study of viruses and their relation to host cell has produced numerous reports on alterations of host cell metabolism due to virus infection. The most prolific source of information has arisen from investigations concerned with effects of bacteriophage on its host cell, and these have been furthered by use of radioactive isotopes (1-3). Similar investigations in the field of animal viruses have not always been so easily resolved as those with bacteriophage, largely due to heterogeneous mixture of cells in host tissue and the difficulty in distinguishing the direct effect produced by virus on infected cells from indirect effects on neighboring cells and the organism as a whole (*e.g.,* inflammation). Anderson, *et al.*(4), using radioactive phosphate, reported that infection of monkey brain tissue with Lansing poliomyelitis virus produced significant changes in rate of phosphate turnover in infected areas of the brain, and suggested that it was due to an increased metabolic activity. Since these changes were detected in specific activities of total acid-soluble organic as well as in inorganic phosphate fractions, the authors discounted the possibility that these changes could be due to injury. No significant alterations in transfer rates from blood to spinal fluid were observed. On the other hand, Rafelson, *et al.*(5), found no changes in turnover of organic acid-soluble phosphates of day-old mouse brain tissue cultures infected with Theiler's GD VII virus which could be related to virus synthesis, although changes in other phosphate fractions attributable to virus synthesis were observed.

It therefore seemed of interest to investigate the effect of a neurotropic virus on the phosphate turnover in infected mouse brain tissue *in vivo* to determine if the effect of the virus infection was due to an actual metabolic requirement for virus synthesis or merely to an increased rate of transfer associated with inflammation.

Materials and methods. Swiss albino mice

* This and related studies constituted thesis submitted by senior author to the Department of Bacteriology, University of Utah College of Medicine, Salt Lake City, in partial fulfillment of the degree of Doctor of Philosophy. This research was supported in part by contract with U. S. Atomic Energy Commission.

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weighing 10 to 15 g (3 to 5 weeks old) were used, and were matched evenly for weight and age in each experiment. The animals were infected by intracerebral inoculation with New Jersey strain of Eastern equine encephalomyelitis (E.E.E.) virus‡ in doses of 0.03 ml. The infected animals were observed for 10 days after injection, although symptoms of infection usually developed within 36 to 72 hours. *Virus titrations.* At appropriate intervals following infection, mice used for determination of virus growth were sacrificed in groups of 5 by exsanguination and the brains rapidly removed, weighed and stored at −26°C until the end of the experiment. Virus concentration for each sample group was determined by 10-fold serial dilution method, employing 5 mice for each dilution per virus sample. LD_{50} was calculated by the method of Reed and Muench(6). *Phosphate determinations.* Mice were injected intraperitoneally with radioactive phosphate ($Na_2HP^{32}O_4$)§, 175 μc/dose, volume 0.1 ml 90 minutes before blood sample was taken by cardiac puncture. The animals were then sacrificed as above and brains rapidly removed. The tissues were dropped into acetone chilled with dry ice, weighed, and homogenized with cold trichloroacetic acid. The homogenates were centrifuged in the cold until a clear supernatant was obtained for analysis. Analytical procedure was that of Ernster, *et al.*(7), which permits estimation of both phosphorus content and radioactivity on same sample. Colorimetric determinations were made with Coleman Jr. spectrophotometer at 625 mμ and radioactivity (P^{32}) was measured with SC1C Tracerlab scaler. Brain tissues of 3 mice were pooled for each sample. Three aliquots were analyzed from each sample, and two samples were used for each result. Results are presented as relative specific activities (R.S.A.) of measured fractions(8). The ratios used were:

$$\text{R.S.A. of orthophosphate (O.P.)} = \frac{\text{Sp. act. brain O.P.}}{\text{Sp. act. blood O.P.}}$$

$$\text{R.S.A. of T.A.S. phosphate (T.A.S.P.)} = \frac{\text{Sp. act. brain T.A.S.P.}}{\text{Sp. act. blood O.P.}}$$

$$\text{R.S.A. of O.A.S. phosphate (O.A.S.P.)} = \frac{\text{Sp. act. brain O.A.S.P.}}{\text{Sp. act. brain O.P.}}$$

Drugs. 1. Cortisone acetate‖ (Cortone, Merck) was injected subcutaneously 2.5 mg per mouse in volume of 0.1 ml 2 hours before virus inoculations. 2. A Pseudomonas pyrogen solution¶ (Piromen, Baxter Laboratories) containing 400 μg/ml was used for intracerebral injection in volume of 0.03 ml. 3. Controls were injected with volumes of sterile 0.85% saline equivalent to appropriate active solution administered.

Results. To correlate virus synthesis with phosphate turnover, a number of young mice were inoculated intracerebrally with approximately 300 LD_{50} units of E.E.E. virus and sacrificed at appropriate intervals for phosphate and virus determinations.

E.E.E. virus was found to proliferate rapidly in brain tissue for the first 24 hours after inoculation (Fig. 1) and the logarithmic rate of virus increase during this period is approximately a straight line. The following period, during which animals developed symptoms (30 hours) and were dying (36 hours), showed no marked change in concentration of measurable virus in the tissues.

The rates of phosphate transfer in the ortho and total acid-soluble phosphate fractions parallel each other, with increased rates first becoming apparent at 12 to 24 hour interval and reaching their maxima at 30 hour or symptomatic period. Changes in phosphate turnovers lagged substantially behind the changes occurring in concentration of active virus in the tissues. The sharp drop in trans-

‡ This virus was obtained through the courtesy of U. S. Public Health Service Laboratory, Montgomery, Ala.

§ Radioactive $Na_2HP^{32}O_4$ was obtained from Oak Ridge, Tenn. and used without addition of carrier. Dilution of stock solution gave the specified activity on the basis of radioactive decay curve. No sample was used more than 10 days after shipment.

‖ Cortisone was obtained from Merck & Co., Rahway, N. J.

¶ Piromen was supplied by Baxter Laboratories, Morton Grove, Ill.

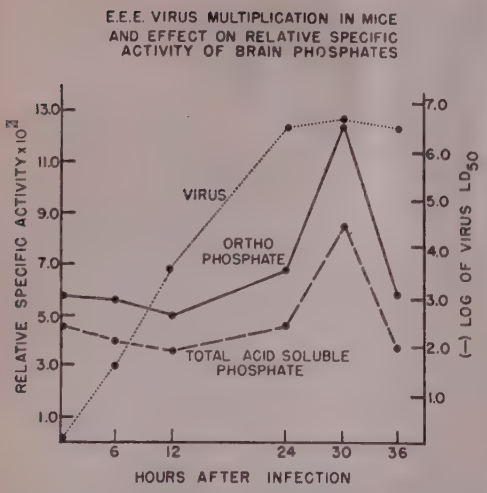

FIG. 1. Relationship of virus synthesis to R.S.A. of phosphate fractions in mouse brain following Eastern equine encephalomyelitis virus infection.

fer rate between 30 and 36 hour periods was probably due to congestion of the brain, which would tend to limit the area of tissue exposed to the P^{32}-containing blood. Marked hemoconcentration of circulating blood and apparent reduction in total blood volume were observed in moribund animals at the 36 hour period.

The organic acid-soluble phosphate (O.A.S.) fraction represents those phosphorylated compounds which are generally assumed to derive their phosphate from the orthophosphate fraction(9). If the virus infection were altering the rate of phosphate uptake on a metabolic level concerned with energy mechanisms of the tissue, it would be evident in the study of this fraction. It is apparent (Table I) that no significant change occurred in the O.A.S. fraction that could be correlated with the remarkable increase in virus concentration which occurred during the 6 to 24 hour interval. However, the R.S.A. of non-infected brain O.A.S. phosphates were again compared to those of virus infected animals to evaluate the apparent decrease noted in Table I. The brain tissues from normal and infected groups, consisting of 15 animals each, were analyzed in groups of 3. Infected animals had been inoculated with the virus 30 hours previously. The results obtained (Table II) indicate that the decrease noted above is not consistent with progression of infection; it may be more readily explained in terms of errors involved in analyses of both ortho and total acid-soluble phosphated fractions, since the O.A.S. represents the difference in both concentration and activity of these two fractions. The progressive nature of the virus infection undoubtedly provokes an increasing inflammatory response in the tissues, and the local change in vascular (blood-brain barrier) permeability associated with this response could account for the increased activity of the ortho-phosphate in brain tissues. Changes in rate of T.A.S. phosphate turnover would then be explained on the basis of increased rate of P^{32} transfer, rather than as increased rate of phosphate utilization.

To evaluate the role of inflammation in alteration of phosphate transfer, 6 groups of

TABLE I. Comparison of Virus Concentration with R.S.A. of Organic Acid-Soluble Phosphate Fraction in Mouse Brain after Infection with Eastern Equine Encephalomyelitis Virus.

Time after infection (hr)	Virus titer	Organic acid-soluble phosphates ($\times 10^1$)
0	$10^{-1.0}$	6.44 (5.65, 7.22)
6	$10^{-1.7}$	4.93 (4.7, 5.15)
12	$10^{-3.6}$	4.12 (3.17, 4.52)
24	$10^{-6.6}$	4.17 (3.98, 4.35)
30	$10^{-6.7}$	4.01 (3.60, 4.42)
36	$10^{-6.5}$	3.24 (2.67, 3.75)

TABLE II. Comparison of R.S.A. of O.A.S. Phosphates from Brain Tissues of Non-Infected Mice with Those from E.E.E. Virus-Infected Animals.

Group* No.	Non-infected (R.S.A. $\times 10^1$)	Virus† (R.S.A. $\times 10^1$)
1	6.22	6.68
2	6.35	5.92
3	7.61	6.76
4	6.66	—
5	5.56	7.59
Avg	6.48	6.73

* Each group consisted of brains from 3 mice.
† Brains obtained from mice infected 30 hr previously with E.E.E. virus.

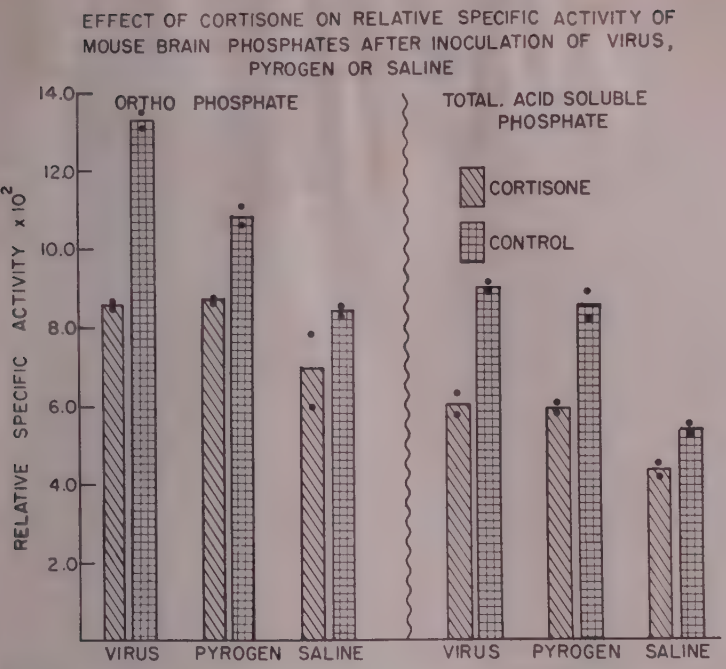

FIG. 2. Effect of cortisone on R.S.A. of mouse brain phosphates after inoculation of virus, pyrogen, or saline.

6 animals each were treated as follows: 3 groups of mice were injected subcutaneously with cortisone, while 3 remaining groups received control injections of 0.85% saline. Two hours later, 6 animals from the cortisone-treated and 6 animals from saline control groups were injected intracerebrally with 30,000 LD_{50} units of E.E.E. virus. At the same time, 6 cortisone-treated and 6 control animals received an intracerebral inoculum of the Pseudomonas pyrogen. The remaining 6 cortisone-treated and 6 saline controls received a control intracerebral injection of 0.03 ml saline. Tissues were obtained for phosphate analysis 28 hours after infection.

Fig. 2 demonstrates quite clearly that increased permeability of the blood-brain barrier to phosphate may be initiated by various agents and is therefore of a non-specific nature. It is interesting that all effects observed in the orthophosphate fraction are reflected in the total acid-soluble phosphate determinations. The hormone treatment effectively modified the increased rate of phosphate uptake in brain tissue injured by either virus infection, pyrogen, or saline injections. Rate of uptake in tissues from animals pretreated with cortisone and subsequently inoculated intracerebrally with virus or pyrogen, showed an R.S.A. no greater than tissue from untreated controls which had received only an injection of saline. It is doubtful, therefore, that increased rate of phosphate transfer serves any useful purpose for synthesis of the virus, but rather, demonstrates the loss of integrity of the blood-brain barrier coincident with the development of inflammation in these tissues.

However, the possibility that the reduced transfer of phosphate might affect the rate of virus synthesis was not wholly disqualified by these experiments. Mice were therefore separated into 2 groups, one of which received 2.5 mg of cortisone subcutaneously. The other group was injected with an equal volume of saline. Two hours later, the animals in each group were injected with approximately 300 LD_{50} units of the virus. Brain tissues from 5 animals of each group were obtained at times noted in Fig. 3 and prepared

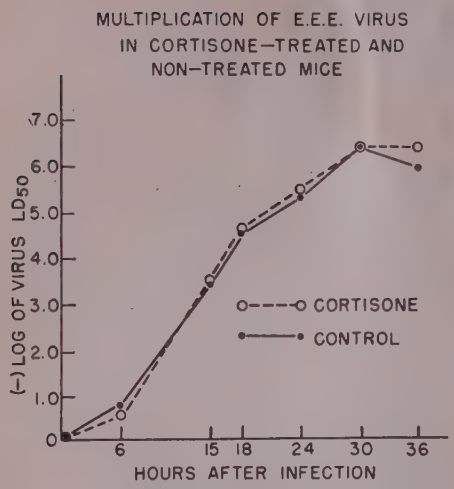

FIG. 3. Synthesis of Eastern equine encephalomyelitis virus in mouse brain of cortisone- and non-treated mice.

for comparative virus determinations. The results show that growth rate of E.E.E. virus in the C.N.S. tissues of the mouse after cortisone treatment is apparently equal to virus growth in those tissues of untreated controls. They suggest that cortisone asserted little or no direct influence on intracellular development of this virus or its ability to infect other susceptible cells of the mouse C.N.S. under the conditions of this experiment.

Discussion. E.E.E. virus infection of mouse C.N.S. tissues was found to enhance the specific activity of brain phosphates following P^{32} administration. Anderson, *et al.*(4), have reported that poliomyelitis virus infection has a similar effect in monkey brain. When our results were analyzed in terms of their *relative specific activities,* the virus effect on phosphates consisted essentially of an enhanced transfer rate of orthophosphate from blood to brain. It has been previously pointed out that a rapid exchange of phosphate normally occurs between the inorganic and acid-soluble phosphates in tissue(9). On this basis, any alteration in transfer rate of P^{32}-labeled inorganic phosphate would be reflected in specific activity of organic phosphates.

Phosphate turnover in degenerating nerve tissue has been observed to be higher than in either normal or regenerating tissue(10), and similarly, P^{32} has been found to accumulate to a greater extent in traumatic, purulent, or ischemic brain lesions than in control tissue (11). A non-specific inflammatory substance (Piromen) was found to substitute for the virus infection in provoking an increased rate of phosphate transfer from blood to brain tissue. Inhibition of this effect by cortisone pretreatment in both virus-infected and pyrogen-treated mice is indicative of a common effect induced by these two different agents. The antiphlogistic effect of adrenal cortical extract and of cortisone has been demonstrated with regard to suppression of alterations in blood-brain barrier permeability(12, 13), as well as of cellular infiltration into virus-infected brain tissue(14).

Anderson, *et al.*(4), suggested that their observed increases in phosphate turnover rates of virus-infected brain tissue must be related to increased metabolic activity within the cell. However, the present study failed to show any correlation between rate of virus growth and turnover rate of organic acid-soluble phosphates. In fact, the only significantly increased activity noted in phosphates depended on increased rate of orthophosphate transfer, and this was shown to follow rather than precede appearance of measurable virus in the tissue. These results suggest that the observed association of increased virus concentration and an altered rate of phosphate transfer is not necessarily indicative of increased metabolic requirements of the virus-infected cell. Cortisone inhibition of the virus effect on rate of phosphate transfer without alteration of rate of virus production implies that the measurable changes demonstrated in this study are evidence of pathology (*i.e.,* inflammation), rather than metabolism.

Summary. Infection of mice with virus of Eastern equine encephalomyelitis induced an enhanced rate of phosphate transfer from blood to brain tissues, which could be markedly reduced by pretreatment of the infected animals with cortisone. Non-specific alteration of phosphate transfer to brain tissue could also be suppressed to a similar degree

by cortisone treatment. Comparison of the E.E.E. virus growth in both cortisone-treated and untreated animals showed no effect of cortisone on rate of virus proliferation. The effect of this neurotropic virus infection on rate of phosphate transfer from blood to brain acid-soluble phosphates appeared to be due to inflammation, rather than to a specific metabolic requirement for virus synthesis.

1. Anderson, J. A., Gemzell, C., Gemzell, L., Bolin, V. S., and Samuels, L. T., PROC. SOC. EXP. BIOL. AND MED., 1950, v73, 690.

2. Evans, E. A., Jr., in *Biochemical Studies of Bacterial Viruses, University of Chicago Press,* 1952, 29.

3. Cohen, S. S., *Fed. Proc.,* 1951, v10, 585.

4. Hershey, A. D., and Chase, M., *J. Gen. Physiol.,* 1952, v36, 39.

5. Rafelson, Max E., Jr., Winzler, R. J., and Pearson, H. E., *J. Biol. Chem.,* 1949, v181, 583.

6. Reed, L. J., and Muench, H., *Am. J. Hyg.,* 1938, v27, 493.

7. Ernster, L., Zetterstrom, R., and Lindberg, O., *Acta Chem. Scand.,* 1950, v4, 942.

8. Hevesy, G., and Hahn, L., *K. danske vidensk. Selsk. Biol. Medd.,* 1940, v15, 5.

9. Dawson, R. M. C., and Richter, D., *Proc. Roy. Soc., B,* 1950, v137, 252.

10. Bodian, D., and Dziewiatkowsky, D., *J. Cell. and Comp. Physiol.,* 1950, v35, 155.

11. Stern, W. E., and Marshall, C., PROC. SOC. EXP. BIOL. AND MED., 1951, v78, 16.

12. Prados, M., Strowger, B., and Feindel, W., *Arch. Neurol. Psychiat.,* 1945, v54, 290.

13. Grenell, R. G., and McCawley, E. L., *J. Neurol. Surg.,* 1947, v4, 508.

14. Aronson, S. M., and Shwartzman, G., *Am. J. Path.,* 1953, v29, 381.

Received March 12, 1956. P.S.E.B.M., 1956, v92.

Electrophoretic Distribution of Serum Proteins in Rabbit, Guinea Pig and Rat Following BCG Administration. (22388)

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Pulmonary tuberculosis in humans has been shown to produce an increase in the alpha-1, alpha-2, and gamma globulin concentration of the serum protein (1-4). Electrophoretic studies of tuberculous guinea pig and rabbit sera revealed an increased total protein and gamma globulin with a decrease in the albumin concentration (5-7).

In an attempt further to understand biochemical factors of host resistance to tuberculosis, a study of serum protein composition in natively resistant (rat) and susceptible (guinea pig and rabbit) animals was undertaken. It also seemed important to study the effect of an immunizing strain (BCG) on the serum protein pattern of susceptible and resistant animals.

Methods. Eight male albino rabbits (avg wt 1100), 8 male albino guinea pigs (550 g) and 8 male albino rats (360 g, Sprague-Dawley) were fasted for 16 hours and bled by cardiac puncture to obtain normal sera. The sera were separated and stored at –20°C until chemical and physical determinations could be made. The animals were then inoculated intraperitoneally with a suspension of tubercle bacilli (BCG).* The rabbits received 6.0 mg; the guinea pigs, 3.0 mg; and the rats, 2.0 mg (wet wt) of the organism suspended in 1.0 ml of saline. The rabbits and guinea pigs were maintained on a diet of rabbit chow pellets (containing no antibiotics) and the rats were fed laboratory Purina chow (no antibiotics). All diets were supplemented with greens and water *ad libitum.* Four weeks following the inoculation of BCG, all of the above animals were again fasted for 16 hours and bled by cardiac puncture. The sera were stored at –20°C. The analyses on control and experimental sera were performed in duplicate at the same time. Electrophoresis of the serum protein was done on

* Culture obtained from Dr. S. R. Rosenthal, Tice Laboratory, University of Illinois, Chicago.

TABLE I. Total Serum Protein Concentrations in Rabbit, Guinea Pig, and Rat following BCG.

Group	Serum total protein g/100 ml	S.D.	"t"
Normal rabbit	7.20	.11	
BCG "	7.67	.21	5.6*
Normal guinea pig	5.68	.09	
BCG " "	6.35	.27	4.6*
Normal rat	6.71	.15	
BCG "	6.67	.13	.6

* Statistically significant (P = 0.01).
S.D. = Stand. dev. from mean.

filter paper using the Durrum(10) type, Spinco, Model R paper electrophoresis apparatus. Whatman 3 MM paper strips 1¼ x 11½ inches were used. All electrophoretic separations were done at room temperature at a constant current (10 Ma) in pH 8.6 veronal buffer (ionic strength 0.075) for 16 hours. The voltage varied with conditions of evaporation within the system. A volume of 0.01 ml of serum was applied with a mechanical applicator on a line 25 mm wide at the apex of the paper strip. At the end of 16 hours of migration the protein was fixed to the paper strips by oven drying at 120°C for 30 minutes. The strips were then stained with bromphenol blue (0.1% in 5% zinc-sulfate-5% acetic acid solution) for 16 hours, rinsed in 5% acetic acid and oven dried for 15 minutes. The protein composition and distribution of the serum was determined by scanning the papers in the Spinco Analytrol Photometer-Computer. Total serum protein was determined by the biuret method of Weichselbaum(11). Absolute values for the protein fractions were computed from the relative distribution and the total protein values. The significance of the deviations from the normal means were computed by the "t" test (12).

Results. The summaries and statistical analysis of the distribution and concentration of serum proteins before and after BCG administration in the rabbit, guinea pig, and rat are presented in Tables I and II.

The administration of BCG resulted in a significant elevation in total protein and gamma globulin in the rabbit and guinea pig but not in the rat. The guinea pig revealed an increase and the rat a decrease in the concentration of alpha-1 globulin. There was a significant decrease in the relative, but not the absolute, concentration of albumin in the rabbit and guinea pig. Significant alterations in the other serum protein components were not observed.

Discussion. The increased total protein concentration in the guinea pig and rabbit following BCG immunization is of interest in light of a report(6) demonstrating a similar protein increase in the tuberculous guinea pig. Similarly, elevation of the gamma globulin has been noted in the tuberculous guinea pig and rabbit(8,9). The possible relationship of the alteration in the alpha-1 globulin composition in the guinea pig and rat to mechanisms of allergy, immunity, or host resistance is not known at this time. The absence of changes in the gamma globulin fraction of rat serum following the inoculation

TABLE II. Electrophoretic Distribution of Serum Protein in Rabbit, Guinea Pig and Rat following BCG.

Group	Albumin		Alpha-1 globulin		Alpha-2 globulin		Alpha-3 globulin		Beta globulin		Gamma globulin	
	Rel. %	g %	Rel. %	g %	Rel. %	g %	Rel. %	g %	Rel. %	g %	Rel. %	g %
Normal rabbit	57.22	4.11	8.73	.63	6.21	.45			14.58	1.06	13.22	.95
BCG "	48.67	3.73	7.85	.60	5.60	.43			14.47	1.11	23.42	1.80
"t" values	6.10*	2.65	1.80	.75	1.50	.57			.20	.94	12.00*	13.90*
Normal G. pig	40.19	2.28	6.45	.36	23.70	1.36	8.75	.50	12.26	.69	8.66	.49
BCG " "	34.32	2.19	7.40	.47	22.53	1.44	8.22	.52	11.97	.76	15.54	.98
"t" values	5.70*	1.18	2.30	2.94*	1.50	1.23	1.00	.58	.40	1.62	9.00*	6.80*
Normal rat	28.89	1.94	16.22	1.09	12.11	.81	5.02	.34	22.00	1.48	15.71	1.06
BCG "	29.70	1.98	14.68	.98	11.87	.79	4.34	.29	22.08	1.48	17.11	1.13
"t" values	1.20	.69	4.00*	4.40*	.60	2.50	1.90	2.00	.20	.00	2.10	1.20

* Statistically significant (P = 0.01).
Rel. % = Percent distribution of total protein. g % = Absolute value for grams of protein/100 ml serum based on total protein.

of an antigen such as BCG is not surprising in view of the fact that this species is known to respond only slightly, or not at all, to most antigenic or allergenic stimuli. It is possible that the unusual lack of response of the rat to BCG immunization is another reflection of its peculiar biological behavior and may be in some way related to its native resistance to infection.

The increase in gamma globulin during BCG immunization in the rabbit and guinea pig follows the classical relation of an increase in gamma globulin with the production of antibody(13,14). The extra-normal T component sometimes seen to occur as a result of certain antigenic stimulation(15) was not observed in this study.

The comparative protein response of the natively resistant rat and susceptible rabbit and guinea pig to tuberculous infection is now under investigation in this laboratory.

Summary. Total serum protein concentration and electrophoretic distribution of the serum protein components of the rabbit, guinea pig, and rat were determined by a paper electrophoretic technic. The serum protein was again analyzed 4 weeks after the animals were inoculated with an attenuated strain (BCG) of tubercle bacilli. Statistically significant increases in the total protein and gamma globulin occurred in the rabbit and guinea pig, but not in the rat. A decrease in the alpha-1 globulin in the rat and an increase of this fraction in the guinea pig was observed following BCG administration. Significant alterations in the other protein fractions were not observed in any of the 3 species of animals.

1. Gutman, A. B., *Advances in Protein Chemistry*, 1948, v4, 212, Academic Press, Inc., New York.

2. Volk, B. W., Saifer, A., Johnson, L. E., and Oreskes, I., *Am. Rev. Tuberc.*, 1953, v67, 299.

3. Saifer, A., Oreskes, I., and Volk, B. W., *ibid.*, 1954, v70, 334.

4. Baldwin, R. W., and Hand, C. N., *ibid.*, 1953, v68, 372.

5. Seibert, F. B., and Nelson, J. W., *J. Biol. Chem.*, 1942, v143, 29.

6. Weimer, H. E., Redlich-Moshin, J., Boak, R. A., Bogen, E., and Carpenter, C. M., *Am. Rev. Tuberc.*, 1954, v70, 344.

7. Johansson, G. A., *Klin. Wchnschr.*, 1949, v27, 70.

8. Meguro, H., and Morikawa, K., *Jap. J. Tuberc.*, 1954, v2, 229.

9. Tsurumi, S., Okada, H., Nishitani, T., Asano, M., and Onishi, S., *Tokyo Med. J.*, 1951, v68, 9.

10. Durrum, E. L., *J. Am. Chem. Soc.*, 1950, v72, 2943.

11. Weichselbaum, T. E., *Am. J. Clin. Path.*, 1946, v16, techn. sec. 40.

12. Hill, A. B., *Principles of Medical Statistics*, 1949, v4, 245, The Lancet Ltd., London.

13. Van der Scheer, J., Lagsdin, J. B., and Wyckoff, R. W. G., *J. Immunol.*, 1941, v41, 209.

14. Tiselius, A., and Kabat, E. A., *J. Exp. Med.*, 1939, v69, 119.

15. Van der Scheer, J., Bohnel, E., Clarke, F. H., and Wyckoff, R. W. G., *J. Immunol.*, 1942, v44, 165.

Received March 12, 1956. P.S.E.B.M., 1956, v92.

Oxidation of Lecithin and Sphingomyelin by Tissue Preparations.* (22389)

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The metabolic functions of the phospholipids are not clearly understood at the present time. Liver(1-3) and liver mitochondria (3) are known to contain dehydrogenases which act on purified phospholipids. However, the ability of other tissues to attack phospholipids has not been tested. Accordingly in the present experiments the phos-

* This work was supported by Grant No. B679 of the National Institutes of Health, U. S. Public Health Service, Department of Health, Education and Welfare.

pholipid dehydrogenase activity of homogenates of brain, liver, skeletal muscle, spleen, and intestinal mucosal was assayed with purified lecithin and sphingomyelin as substrates. The cofactors added in these experiments were hypoxanthine, ATP, or DPN, since these compounds have been found necessary for known phospholipid(1-3) or fatty acid dehydrogenases(4-6).

Methods. The sources and purity of the phospholipids and other chemicals used in this study have been previously described(3). Homogenates of rat skeletal muscle were prepared by mixing 5 g of rat spinal muscle with 50 ml of 0.25 M sucrose for 1 minute at 0° in the 125 ml cup of the Waring blendor at ⅓ of full speed. Homogenates of other tissues were made by grinding 1 part of finely divided tissue with 2 parts of 0.25 M sucrose in a motor driven all glass homogenizer(7). All preparations were filtered through fine cheese cloth before use. Acetone powders were prepared in the usual manner from suspensions of liver mitochondria in 0.25 M sucrose or from homogenates of intestinal mucosa. Extracts of the acetone powders were made by grinding 1 part of fresh powder with 3 volumes of isotonic sucrose and centrifuging at 18,000 G at 0° for 30 minutes. Rat liver mitochondria were prepared by the method of Kielley and Kielley(8) except in the cases of those organelles used for acetone powders. The mitochondria used for the latter purpose were isolated on a large scale by the method of Witter, Pories, and Cottone (9). In each case the particulates were suspended in enough 0.25 M sucrose so that 1 ml of suspension was equivalent to the mitochondria from 0.5 g of liver. In a total volume of 1 ml the complete reaction mixture contained 0.1 mg of substrate, 0.01 M phosphate buffer pH 7.4, 0.025 M sucrose, 0.01 M sodium malonate, 0.01 M potassium cyanide, 0.005% 2,6-dichlorobenzenoneindo-3′-chlorophenol, either 0.001 M ATP or 1 x 10^{-4} M DPN, and either 0.4 ml of homogenate of rat spinal muscle or 0.1 ml of the homogenate of other tissues or extracts of acetone powders. In some experiments 0.005 M magnesium chloride was added. Under anaerobic conditions 0.0033% methylene blue could replace the indophenol dye plus cyanide. The dehydrogenase activity was estimated by comparing the rate of reduction of the dye in a tube containing the complete reaction mixture with that in another tube containing a similar mixture from which the substrate had been omitted. The reduction of the dye was followed for 1 hour.

Results. In Table I are given the results of the studies of the phospholipid dehydrogenase activity of homogenates of spleen, muscle, intestinal mucosa and suspensions of mitochondria from rat liver, using hydrolecithin or sphingomyelin as substrates. For purpose of brevity data showing that the systems are inactive in the absence of added DPN or ATP have been omitted. It can be seen that at least 2 enzymes or enzyme systems were detected. The first of these, a dehydrogenase requiring ATP, was found in liver mitochondria as previously described(3) but in addition was observed in spleen. Other experiments showed that the dehydrogenase of mitochondria was inhibited by the concentration of magnesium chloride employed whereas the enzymes in the other homogenates were not. The second type of dehydrogenase required DPN and was present in muscle, intestinal mucosa, and spleen. The enzyme in the latter tissue may be different from the one present in the intestinal mucosa or muscle since the dehydrogenase of spleen acted only on sphingomyelin while that of intestinal mucosa or muscle acted either on lecithin or sphingomyelin. Other experiments not given in Table I showed that homogenates of whole liver did not reduce the dye at an increased

TABLE I. Distribution of Phospholipid Dehydrogenases.

Assay system ATP	DPN	Substrate	Reduction time in min. Liver mitochondria	Spleen	Muscle	Intestine
+	—	0	11	>60	21	30
+	—	Hyd†	3	9	24	25
+	—	Sph	6	>60	23	>60
—	+	0	8	>60*	19*	14
—	+	Hyd	9	>60*	6*	6
—	+	Sph	8	3*	9*	7

* Magnesium chloride was present.
† Hyd = Hydrolecithin; Sph = Sphingomyelin.

rate in the presence of DPN and that neither the dehydrogenase which required DPN nor the one for which ATP is a cofactor could be detected in homogenates of brain or kidney. The only tissue in which the phospholipid dehydrogenase which requires hypoxanthine(1, 2) could be detected was liver, and in confirmation of previous work(3) this dehydrogenase was not present in the liver mitochondria.

The phospholipid dehydrogenase of intestinal mucosa was found to be present in an acetone powder of this tissue as had previously been reported for the enzyme of rat liver mitochondria(3). In addition the phospholipid dehydrogenase of liver which required hypoxanthine was inactivated by treatment with acetone. Thus acetone powders of whole liver could also be used as a source of the mitochondrial enzyme. As is shown in Table II the enzymes could be obtained in soluble form from the acetone powders.

In the next experiment the specificity of the phospholipid dehydrogenase present in the extracts of acetone powder of intestinal mucosa or liver mitochondria was investigated. Oleic acid, lecithins containing both saturated and unsaturated acids, and lipids containing sphingosine such as sphingomyelin, cerebroside, or ceramide were active. Evidently the action of these enzymes is not limited to the catalysis of dehydrogenation in the 9, 10 position of the fatty acid moiety. It is of interest that the synthetic lipid glycolecithin which has not been found in natural products was attacked by the enzyme system.

TABLE II. Extraction of Phospholipid Dehydrogenase from Acetone Powders of Liver and Intestine.

	Reduction time in min.			
	Intestine		Liver mitochondria	
Enzyme source	Endogenous	Hydrolecithin	Endogenous	Hydrolecithin
Original powder	12	5	10	5
Residue from centrifugation	>60	>60	>60	>60
Supernatant	29	11	18	7
Dialyzed supernatant	>60	>60	>60	>60

TABLE III. Specificity of Phospholipid Dehydrogenase in Extracts of Acetone Powders of Liver Mitochondria and Intestine.

	Reduction time in min.	
Substrate	Liver mitochondria	Intestine
None	13	16
Sphingomyelin	7	9
Ceramide	8	10
Palmitoleyl lecithin	4	7
Hydrolecithin	4	7
Cerebroside	7	10
Oleic acid	4	10
Palmitic acid	3	10
Glycolecithin	9	11

The results of these studies show that the rate of reduction of indophenol dyes by homogenates of various tissues is increased by the presence of lecithin and sphingomyelin. The exact nature of the reaction which causes the reduction of the dye is not revealed by these experiments. Rodbell and Hanahan(10) have shown that small amounts of lecithin and its derivatives will catalytically stimulate the respiration of liver homogenates supplemented with acids of the Krebs cycle. However, this does not seem to be the case in the present experiments since the endogenous respiration was repressed with malonate, since specific cofactors were required and since no increase in the rate of reduction was noted if the level of phospholipid was reduced to values less than ¼ that usually employed. It is more probable that the increased rate of reduction of the dye in the present experiments is a measure of the oxidation of either the intact phospholipid or its hydrolysis products. In view of the differences in the effect of the presence of magnesium chloride and in the requirement for activating factor for each tissue it appears probable that the reactions measured by the phospholipid dehydrogenase system are not the same in each tissue.

Investigation of the products of these reactions by means of paper chromatographic methods for phospholipids and their hydrolysis products which were recently developed in this laboratory(11) may reveal the exact pathway by which the phospholipids are attacked in each system. The present experiments have provided a basis for the further study of the oxidation of phospholipids *in*

vitro since four tissues worthy of further investigation have been ascertained, and the active enzymes have been obtained in soluble form from two of them.

Summary. Liver mitochondria and spleen were found to contain dehydrogenases which attacked lecithin and sphingomyelin and required ATP. Muscle and intestinal mucosa had dehydrogenase activity with lecithin or sphingomyelin if DPN was present whereas under the same conditions homogenates of spleen were active only with sphingomyelin. The dehydrogenase systems were obtained in soluble form from acetone powders of liver mitochondria or intestinal mucosa.

1. Annau, E., *Enzymologia*, 1941, v9, 150.
2. Annau, E., Eperjessy, A., and Felszegly, G., *Z. Physiol. Chem.*, 1942, v277, 58.
3. Marinetti, G. V., and Stotz, E., *J. Biol. Chem.*, 1955, v217, 745.
4. Lang, K., and Mayer, H., *Z. Physiol. Chem.*, 1939, v261, 249.
5. Burton, K., *Nature*, 1948, v161, 606.
6. Blakley, R. L., *Biochem. J.*, 1952, v52, 269.
7. Dounce, A. L., and Beyer, G. T., *J. Biol. Chem.*, 1948, v174, 859.
8. Kielley, W. W., and Kielley, R. K., *ibid.*, 1951, v191, 485.
9. Witter, R. F., Pories, W. W., and Cottone, M. A., PROC. SOC. EXP. BIOL. AND MED., 1953, v84, 674.
10. Rodbell, M., and Hanahan, D. J., *J. Biol. Chem.*, 1955, v214, 595.
11. Rouser, G., Marinetti, G., Witter, R. F., Berry, J. F., and Stotz, E., *ibid.*, in press.

Received March 21, 1956. P.S.E.B.M., 1956, v92.

Phenylserine Studies. IV. Biochemical Activity of *o*- and *p*-Fluorophenylserine. (22390)

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Phenylserine was shown(1) to be an inhibitor of the growth of various species of bacteria. The inhibition was associated with certain enzymatic sequences in phenylalanine and tryptophan metabolism, since either of these two amino acids was able to reverse the inhibitory effect. Subsequently, the discovery of chloromycetin, which may be considered to be a phenylserine derivative, has stimulated increased interest in the bacteriostatic action of this type of compound(2). Fluoro-derivatives of various metabolites have frequently proven of interest in the study of metabolic sequences, presumably because the small atomic dimension of the fluorine atom does not prevent the reaction of the analogue with an enzyme, but does prevent completion of the enzymatic sequence. The fluoro-analogues of phenylalanine have previously been prepared and tested as to their bacteriostatic properties(3,4). It therefore seemed of interest to synthesize and test the properties of ring-substituted fluoro-derivatives of phenylserine. The present paper describes the microbiological effects of the *o*-fluoro- and *p*-fluoro- analogues of phenylserine.

Materials and methods. Fluorophenylserines. Into an evaporating dish are introduced 19.8 ml of a 2.5 M glycine solution, 9.9 ml of 95% ethyl alcohol, 9.0 ml of *o*- or *p*- fluorobenzaldehyde,* and 19.8 ml of 6.25 M sodium hydroxide. The mixture is stirred until it becomes clear, permitted to stand for one hour or until a waxy white precipitate forms, and the precipitate dried between sheets of filter paper. The precipitate is washed twice with 15 ml portions of ethanol and then twice with 10 ml portions of water. To the residue is added 500 ml of water with stirring, acetic acid is added to pH 6.0, and any residue is filtered off and discarded. The filtrate is evaporated to dryness, taken up in hot water, and recrystallized from the water

* Obtainable from Custom Chemical Laboratories, Chicago, Ill.

in the ice box. The crystals are dried between sheets of filter paper, and then in a vacuum desiccator over calcium chloride. Paper chromatography demonstrates in each case that a new amino acid has been formed almost quantitatively from the reactants. The *m*-fluoro- analogue is not readily prepared by this procedure.

o-fluorophenylserine, mpt. 178°C.

$C_9H_{10}O_3NF$ (199).

Calculated, N 7.04; found, N 7.67
Calculated, C 54.27; found, C 55.99
Calculated, H 5.02; found, H 5.66

p-fluorophenylserine, mpt. 193°C.

$C_9H_{10}O_3NF$ (199).

Calculated, N 7.04; found, N 7.18
Calculated, C 54.27; found, C 55.06
Calculated, H 5.02; found, H 5.28

Microbiological Assay. Neither the *o*- nor *p*-fluorophenylserine produces a visible precipitate with any of the recognized trace elements (5). They produce no observable effect on *Aspergillus niger* or *Serratia marcescens.* Against *Escherichia coli* and using previously described methods(1), only *p*-fluorophenylserine was inhibitory. The nature of this inhibition is described in detail in the following paragraphs.

Results. Complete inhibition of the growth of *Escherichia coli* with *p*-fluorophenylserine was achieved at levels of approximately 30 μg per ml, with 50% inhibition at 20 μg per ml. When an extensive series of metabolites was tested for reversal effects against *p*-fluorophenylserine, only tyrosine, phenylalanine, and tryptophan showed measurable effects. The effects of varying levels of these amino acids in reversing the *p*-fluorophenylserine inhibition are shown in Table I. A yeast extract solution reversed the inhibitor at levels equivalent to a phenylalanine content of five percent. Paper chromatography of the extract failed to show any fraction with activity other than the amino acids previously mentioned.

Discussion. The principles governing the elucidation of metabolic sequences on the basis of data obtained from inhibition studies have previously been described(6). Experience with phenylserine(1) and *p*-fluorophenylalanine(4) have indicated that the inhibitory action of both of these compounds is reversed by phenylalanine and tryptophan, and in some cases by tyrosine. Bergmann *et al.*(4) have analysed these data, and extended their significance by the study of similar relationships in phenylalanineless, tyrosineless and tryptophanless mutants of *Escherichia coli.* From these studies it was concluded that tryptophan acts as a phenylalanine precursor, and that phenylalanine acts as a tyrosine precursor, but that an alternate synthetic pathway exists between tryptophan and tyrosine.

TABLE I. Effect of Phenylalanine, Tyrosine, and Tryptophan in Reversing the *p*-Fluorophenylserine Inhibition of *Escherichia coli.*

Reversing agent (μg/ml)	*p*-Fluorophenylserine (μg/ml) 0	30	100	300	1000
	% maximum growth				
Control	100	0	0	0	0
L-Phenylalanine					
.003	100	60	0	0	0
.010	100	100	25	5	0
.030	100	100	100	55	0
.100	100	100	100	100	55
.300	100	100	100	90	50
L-Tryptophan					
10	100	20	0	0	0
30	100	70	15	0	0
100	100	85	70	15	0
300	100	100	100	65	60
1000	100	100	100	100	100
L-Tyrosine					
10	20	20	20	15	
30	20	20	15	15	
100	40	45	50	45	
300	40	35	30	10	

In the present study phenylalanine has been found to be approximately 10,000 times as effective as tryptophan in reversing the toxicity of *p*-fluorophenylserine. Tyrosine is roughly as effective as tryptophan, but since tyrosine is itself toxic to this strain of *Escherichia coli*(7), synergistic inhibitory effects appear and it is impossible to obtain consistent data. The data thus seem to support the straight line precursor relationship previously proposed between tryptophan, phenylalanine and tyrosine. The fact that large amounts of phenylalanine also tend to reverse the inhibition of microorganisms by 5-methyltryptophan suggests that the relationship between tryptophan and phenylalanine is a reversible one.

Summary. The *o*- and *p*-fluoro- analogues of phenylserine have been synthesized and tested for their inhibitory effects on bacterial growth. Only *p*-fluorophenylserine inhibits the growth of *Escherichia coli*, and its action is reversed by phenylalanine, tryptophan, and to a limited extent, tyrosine. These results seem to emphasize the existence of a phenylalanine-tryptophan metabolic interconversion in bacteria.

1. Beerstecher, E., Jr., and Shive, W., *J. Biol. Chem.*, 1946, v164, 53.

2. Beerstecher, E., Jr., Edmonds, E. J., and Volkmann, C., *Texas Rep. Biol. Med.*, 1955, v13, 195.

3. Martin, G. L., and Mon, L. N., *Am. J. Pharm.*, 1949, v121, 169.

4. Bergmann, E. D., Sicher, S., and Volcani, B. E., *Biochem. J.*, 1953, v54, 1.

5. Volkmann, C. M., and Beerstecher, E., Jr., *J. Bact.*, 1955, v70, 476.

6. Shive, W., and Macow, J., *J. Biol. Chem.*, 1946, v162, 451.

7. Beerstecher, E., Jr., and Shive, W., *J. Biol. Chem.*, 1947, v167, 527.

Received March 26, 1956. P.S.E.B.M., 1956, v92.

Transportation of Human Cells Cultured *in vitro*.* (22391)

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The increased use of tissue cultures for studies in biology and medicine has made essential the transportation of living animal cells. Although cell cultures have occasionally been transported between laboratories, *e.g.*, mouse, strain L cells(1), detailed information and optimal conditions for shipment of cells are not on record. The extensive application of a strain of human epithelial cells, strain HeLa (Gey), to studies of poliomyelitis virus(2,3) made desirable the preparation of HeLa cell cultures in a central laboratory for distribution to virus laboratories. Preliminary studies showed that strain HeLa cells withstand shipment by ordinary train or plane(2). Since this information was inadequate for mass distribution of cultures from the central laboratory established at Tuskegee Institute, Ala., the following experiment was performed. The results of this experiment pertain to one type of cell; data describing shipment of another cell-type, *i.e.*, monkey kidney cells, has recently been reported(4). It is hoped that methods for transportation of other cells will soon be recorded.

* Aided by grants from National Foundation for Infantile Paralysis.

Materials and methods. Cultures of strain HeLa cells on glass were prepared 2-5 days before shipment, by transfer of cells in a fresh mixture of adult human serum, 40% and Hanks's balanced salt solution, 60%, (HuS-40, H-60), from stock bottle cultures to screw capped 16 x 125 mm test tubes. Each tube contained 70,000 to 100,000 cells at the beginning of shipment; 1341 tube cultures were sent.

Results. The experiment was designed on the premise that the following 6 factors relate fundamentally to successful transportation of animal cells and thus the experimental results are discussed in relation to each factor.

1) The *nutritional status* of cells at the beginning of shipment was evaluated by comparing survival of cells a) placed in fresh HuS-40, H-60 one day before shipment, and b) kept for 2-5 days in the HuS-40, H-60 medium used for preparation of the cultures. The experimental results (Table I) show that placement of cells in fresh medium for 1 day prior to shipment increased significantly the percentage of surviving cultures.

2) The *availability of nutrients* in relation to nutritional needs of cells during shipment and to a limited extent, 3) *mechanical trauma* to cells was studied by sending cells with and

TABLE I. Transportation of Strain HeLa Cells: Effects of Nutritional Status of Cells, Medium during Shipment, and Duration of Shipment.

Days between change of medium and shipment	Medium* during shipment	Cultures satisfactory† after shipment: Results by days in transit 1 Ratio‡	%	2 Ratio	%	3 Ratio	%	4 Ratio	%
1	Yes	124/142	87	65/69	94	88/122	72	62/122	51
1	No	69/ 69	100			67/112	60	50/120	42
2–5	Yes	8/ 69	12			0/175	0	0/ 46	0
2–5	No	50/ 71	70			96/178	54	1/ 46	2

* Medium = adult human serum, 40%, and Hanks' balanced salt solution, 60%.

† Satisfactory signifies a tube culture with at least 50,000 cells of normal morphology, 24-48 hr after arrival and incubation at 36°C. HuS-40, H-60, 0.5 ml, was added on arrival to cultures sent without medium.

‡ Numerator = No. of satisfactory cultures; denominator = No. of cultures shipped.

without liquid medium in the tubes. Supernatant liquid was neither essential nor detrimental to survival of well-nourished cells, *i.e.*, those fed 1 day before shipment (Table I). Contrariwise, survival rates for less well-nourished cells, *i.e.*, those fed 2-5 days before shipment, were lower in cultures with liquid medium because the cells were often detached from the glass evidently by movement of the fluid.

4) The *duration of shipment* was varied from 1-4 days by employing for shipment between Tuskegee Institute, Ala. and Minneapolis, Minn., air parcel post, special delivery for one-day delivery, air parcel post for 2-day delivery and regular parcel post for 3- and 4-day delivery. Strain HeLa cells survived periods of one or 2 days in transit better than 3 or 4 days (Table I).

5) *Temperature* as a factor in transportation was difficult to study in detail. Despite efforts to keep the temperature between 15° and 30°C by employing insulated cardboard boxes, temperatures recorded by maximum-minimum temperature thermometers for several shipments during the period February and March 1954 ranged from 5-20°C. During the summer, cells died in several boxes where the maximum temperature was 43°C; during cold weather cells were destroyed by freezing.

6) The *method for revival of cells* following transportation is obviously important and must furnish cells with adequate nutrients at a proper temperature. A standard procedure was employed in this experiment to revive cells after shipment, *i.e.*, each culture was incubated at 36°C for 24-48 hours and HuS-40, H-60, 0.5 ml, was added to cultures sent without medium. This procedure brought the cells within 24-48 hours into a healthy state (*i.e.*, thin delicate membranes, clear agranular cytoplasm, polygonal shape and evidence of acid production by change in color of phenol red).

Discussion. The results of this experiment revealed that survival of strain HeLa cells shipped by train or plane was enhanced: a) by placement of cells in fresh medium 1 day before shipment, b) by completion of travel within 2 days, and c) by protection of cells from excessive heat and from freezing. Moreover, the lack of a detrimental effect of supernatant liquid upon cells well-nourished at the beginning of shipment made feasible the shipment of medium in the cultures to avoid the laborious addition of medium to each tube after shipment.

With this information, the laboratory at Tuskegee Institute proceeded with the shipment of large quantities of HeLa cell cultures. Tube cultures each containing 70 to 100 thousand cells at the beginning of shipment and bottle cultures with 5 to 10 million cells, received fresh medium (HuS-40, H-60) 1 day before shipment. The medium was left in the tubes during transit but removed from the bottle cultures since fluid tended to detach from glass the sheets of closely packed HeLa cells in bottle cultures and because cells shipped as well without as with medium (Table I). Shipments were completed within 3 days by air parcel post, air express or regular parcel post. During warm weather, *i.e.*,

April to September, one or 2 cans of sodium sulfate decahydrate ("Equitherm")(5) were enclosed in each package to help keep the temperature near the cultures below 38°C.

During the period April 1 through Sept. 30, 1954, approximately 133,000 tube cultures and 1,800 bottle cultures were sent from Tuskegee Institute to laboratories in 23 cities located in all sections of the United States. Although it was not possible to determine precisely the outcome of each tube and bottle, reports from recipient laboratories indicated that more than 90% of the cultures were received in satisfactory condition. By application of the principles established in this experiment, large numbers of strain HeLa cells in suspension were shipped and successfully used for detection of neutralizing antibodies for poliomyelitis virus(6). Since the percentage of successful shipments may be influenced by factors such as seasonal temperature, only further experience in shipment will reveal the extent to which this percentage fluctuates.

The conclusions derived from these studies that may be applicable to transportation of other animal cells follow: a) Cell survival is related inversely to duration of travel. b) Cell nutritional status at the beginning of shipment is a critical factor; placement of strain HeLa cells in fresh medium for a day before shipment significantly increased survival. c) The temperature of cells during shipment should be controlled to prevent death of cells from excessive heat or cold and to keep metabolic requirements of cells at a level met by the supply of nutrients. Evidence that cells can be preserved at subzero temperatures(7) may make it feasible to keep cells during transit at the temperature of storage. d) The nutritional requirements for maintenance of cell viability must be met during transit. For example, the nutrients needed by strain HeLa cells, well-nourished at the beginning of 1-2 day shipments, at temperatures that assure low metabolic rates were supplied by the few residual drops of medium left after removal of the supernatant fluid. For longer periods in transit or for other types of cells, a larger supply of nutrients may be essential for survival. However, as cell damage increases with duration of shipment, mechanical trauma to cells from movements of supernatant liquid may result in detachment and death of cells. Indeed, for strain HeLa cells in transit for 3 or more days, limitation of the supernatant liquid to several drops seems advisable. Alternatively, cells could be embedded in plasma or in gel foam(8) to prevent detachment from glass and to keep nutrients near cells.

Summary. Living human epithelial cells, strain HeLa (Gey), readily survive transportation over periods of 1-3 days. Six factors in survival, *i.e.*, 1) nutritional status of cells at the beginning of shipment, 2) availability of nutrients in relation to nutritional needs of cells during shipment, 3) mechanical trauma to cells, 4) duration of shipment, 5) temperature near cells and 6) the method for revival of cells following shipment, were studied to define satisfactory conditions for routine shipment of strain HeLa cells.

1. Earle, W. R., personal communication.

2. Scherer, W. F., Syverton, J. T., and Gey, G. O., *J. Exp. Med.*, 1953, v97, 695.

3. Syverton, J. T., Scherer, W. F., and Ellwood, P. M., *J. Lab. and Clin. Med.*, 1954, v43, 286.

4. Melnick, J. L., Rappaport, C., Bank, D. D., and Bhatt, P. N., PROC. SOC. EXP. BIOL. AND MED., 1955, v88, 676.

5. Thermally insulated package. *Chemical and Engineering News*, 1954, v32, 3380.

6. Lipton, M. M., and Steigman, A. J., PROC. SOC. EXP. BIOL. AND MED., 1955, v90, 367.

7. Scherer, W. F., and Hoogasian, A. C., *ibid.*, 1954, v87, 480.

8. Leighton, J., and Kline, I., *Texas Rep. Biol. and Med.*, 1954, v12, 865.

Received March 26, 1956. P.S.E.B.M., 1956, v92.

Cultivation and Transmission of Etiological Agent of Kidney Disease in Salmonid Fishes. (22392)

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During recent years there have been increasing numbers of outbreaks of a condition called "kidney disease" in stocks of young salmon in hatcheries in the Pacific Northwest (1). In many cases catastrophic mortalities have resulted(1). The disease has been found in adult salmon on the spawning migration (2). Kidney disease has also been found in stocks of trout in hatcheries in the Pacific Northwest, and serious outbreaks of the disease have been reported in trout in Eastern United States(3,4). The histopathology of kidney disease in trout and salmon has been compared and found to be almost identical (5). Outbreaks of disease apparently similar to or identical with kidney disease have been reported in Atlantic salmon in Scotland(6) and in rainbow trout in Germany(7).

Tremendous numbers of tiny Gram-positive organisms often in pairs are characteristically found both intracellularly and extracellularly in the lesions of infected fish. These have been commonly referred to as diplobacilli. Except in its earliest phases, kidney disease in salmon and trout can be readily diagnosed by its pathology supplemented by examination of sections or smears from the diseased tissues stained by Gram's method. Nevertheless, studies on the disease and the nature of the etiological agent have been handicapped by a lack of satisfactory media for direct isolation and cultivation of the etiological agent. Apparently this difficulty has occurred in all laboratories where kidney disease has been studied. A number of investigators have failed in their attempts to cultivate the etiological agent(3,4,6,7). It has even been suggested that the organism is related to the etiological agent of salmon disease of canines(1,3), which is now considered to be one of the Rickettsia.

Methods of cultivation. After a number of unsuccessful attempts, cultivation of the etiological agent of kidney disease was achieved by several different methods. Cultivation of the organism was first achieved in suspensions of minced chick embryo tissues incubated at 15°C, and it was possible to make serial subcultures on this medium(1). However, this system did not prove convenient, and subsequently has been replaced by other methods. In one experiment a culture of the etiological agent of kidney disease, which had been grown on chick embryo tissues, was subcultured on a number of different types of culture media. Among these was Dorset's medium, as used for the cultivation of *Mycobacterium tuberculosis*. After approximately two months of incubation at 15°C, well developed colonies were found on this medium. These colonies contained organisms which were morphologically identical with those occurring in lesions of diseased fish. The bacteria were transferred on Dorset's medium, and after 5 serial subcultures were tested for pathogenicity to young salmon. Typical kidney disease was produced and the etiological agent reisolated from a number of the fish taken either in a moribund condition or shortly after death. In every case huge numbers of characteristic Gram-positive diplobacilli were found in smears taken from the kidneys of the experimental fish. Standard Dorset's medium did not prove satisfactory for the isolation of the etiological agent of kidney disease in natural outbreaks of the disease. A modified Dorset's medium fortified with cysteine, tryptone and yeast autolysate was prepared and subjected to greater heat treatment than is usual in the preparation of this medium. On this modified medium, direct inoculation from lesions of infected fish regularly resulted in good growth of the etiological agent. Serial subcultures were carried out without difficulty, and good growth was obtained in a period of 3 to 4 weeks at 17°C. This modified

Dorset's medium has been found useful in bacteriological diagnosis of kidney disease. Pure cultures were usually obtained except when the fish were dead or secondary bacterial infection had set in. It was found that only a small portion of the bacteria present in the inoculum developed on the modified Dorset's medium. This difficulty has been overcome by the use of a modified blood agar. It was noted that occasionally when kidney tissues from infected fish were streaked on plates of blood agar containing a tryptose base, growth of bacteria occurred in and around bits of kidney tissue. However, colonies did not develop on restreaking these bacteria on the same medium. It was also found that some stock cultures which had been maintained on modified Dorset's medium for a dozen or more transfers would develop when transferred to tryptose base blood agar. In a further search for a suitable plating medium, a variety of nutrient supplements were incorporated into tryptose base blood agar. The essential requirement proved to be cysteine. The addition of 0.05 to 0.1% cysteine to the tryptose base blood agar produced a medium on which growth occurred within 7 to 10 days when streaked directly from infected fish and incubated at 17°C. Somewhat better development occurred when the amount of blood (human) was increased from 10 to 20% by volume, and 0.05% yeast extract was added. The medium now in use is as follows:

	%
Tryptose	1.0
Beef extr.	0.3
NaCl	0.5
Yeast extr.	0.05
Cysteine-HCl	0.1
Human blood (by vol.)	20
Agar	1.5

Characteristic colonies are shown in Fig. 1. There is often a considerable variation in colony size on cysteine blood agar, although the colonies of all sizes contain bacteria of the same morphological characteristics. These are shown in Fig. 2. Cysteine blood agar has also proven satisfactory for the cultivation and isolation of the diplobacilli from fish in early stages of the disease before any characteristic pathology has developed. In this case the incubation period is often prolonged, presumably because the number of bacteria in the inoculum is small. In one experiment designed to test the requirement for cysteine, 10 fish were selected from a population of blueback salmon in which natural kidney disease

FIG. 1. Colonies on cysteine blood agar. Streaked directly from infected tissue. × 5.

FIG. 2. Electron micrograph of diplobacilli from a cysteine blood agar plate. The cells were lightly shadowed with palladium at a 1:5 angle.

was present. None of the fish had been inoculated deliberately. At autopsy 5 of the fish showed gross lesions of kidney disease and 5 appeared normal. Material from the posterior portion of the kidney of each fish was inoculated on 2 blood agar plates lacking cysteine and on 2 blood agar plates in which cysteine was present. All plates were incubated at 17°C and observed at intervals up to 6 weeks. The plates from one fish remained sterile during the entire period of observation. The blood agar plates containing cysteine inoculated from all other fish developed colonies, many of them appearing at 9 days, others as late as 17 days after inoculation. No growth occurred on any of the blood agar plates deficient in cysteine until the 5th week when 1 to 6 colonies were found on each of 4 plates. The remaining 16 plates remained without growth. Whereas subinoculation from the four plates showing colonies to other tryptose blood agar plates deficient in cysteine yielded no growth, good growth occurred on subinoculation into blood medium containing cysteine.

Transmission experiments. The requirements of Koch's postulates as usually formulated have been met with the bacterium cultivated from fish with kidney disease. The bacterium is regularly present in lesions of the disease. Typical kidney disease has been produced by inoculation of pure cultures of the bacterium. Finally, the bacterium has been reisolated from fish with the experimentally produced disease.

In one experiment, cultures of the diplobacilli were isolated from the liver of an adult spring chinook salmon (*Oncorhynchus tshawytscha*). After replating 3 times on cysteine blood agar, 0.05 ml of a dilute suspension of the diplobacilli in saline was injected intraperitoneally into each of 20 yearling blueback salmon (*Oncorhynchus nerka*) held at 15°C. The first deaths occurred on the 12th day and all fish were lost by the 23rd day. The experiment was repeated after 2 further transfers of the culture. In this experiment all except one fish were lost between the 12th and 21st day. This fish survived until the 24th day. Characteristic diplobacilli were found in huge numbers in smears taken from each test fish, and the organisms were reisolated from a number of the fish taken shortly after death.

An additional experiment was carried out in order to compare the virulence of isolated cultures of the diplobacillus with that of organisms maintained in frozen fish tissues. A suspension was prepared from a portion of the liver of the adult spring chinook salmon and injected into a number of yearling blueback salmon. The liver had been kept at −20°C for about 4 months. After mortalities had set in, 3 of the fish were sacrificed. A suspension was made from the organs of these fish and injected intraperitoneally into each of 20 yearling blueback salmon in amounts of 0.05 ml. The fish were held at 15°C. The first death occurred on the 13th day, and all fish were lost by the 20th day. It is apparent from this experiment that the pure cultures grown *in vitro* were comparable in virulence with the organisms preserved in the frozen tissues of infected fish.

It has been our experience that kidney disease in trout is ordinarily less severe than in salmon, even though heavy mortalities have occurred in some trout hatcheries. Diplobacilli isolated from trout in the Pacific

Northwest are indistinguishable from those isolated from salmon, and are comparable in virulence toward salmon. In one experiment a dilute suspension of diplobacilli isolated on cysteine-blood agar from a trout during the course of an outbreak of kidney disease in a trout hatchery, was inoculated into a stock of blueback salmon under conditions similar to those given above. A heavy mortality set in on the 14th day and all fish were lost by the 19th day.

Etiological agent. In view of its morphology and its development on culture media, the etiological agent of kidney disease is clearly one of the bacteria. The organism is a Gram-positive, short rod, and occurs frequently in pairs. The dimensions are variable but most cells range from 0.3 to 0.5 μ by 0.5 to 1.0 μ. Chains are sometimes observed in old laboratory cultures. A considerable degree of pleomorphism has sometimes been noted in intracellular forms in infected tissues and in some laboratory cultures. No endospores or resistant forms have been found. The organism is proteolytic and an active catalase is present. On the basis of present information, the organism may be considered to be a species of *Corynebacterium*. However, further studies on the properties of the organism are being carried out, and the exact taxonomic status is still under study.

Summary. Media have been devised for the cultivation and isolation of the etiological agent of kidney disease in salmon and trout. It is noteworthy that growth occurs readily on addition of cysteine to complex blood media, which otherwise do not support growth. Koch's postulates have been fulfilled establishing that the organism isolated is the etiological agent of the disease.

1. Earp, B. J., Ellis, C. H., and Ordal, E. J., *Wash. Dept. Fish Spec. Rep. Ser.* No. 1, 1953, 74 pp.

2. Wood, J. W., and Wallis, J., unpublished manuscript.

3. Belding, D. L., and Merrill, B., *Trans. Am. Fish. Soc.,* 1935, v65, 76.

4. Snieszko, S. F., and Griffin, P. J., *Prog. Fish-Culturist,* 1955, v17, 3.

5. Wood, E. M., personal communication.

6. Blake, Isobel, Final Report of the Furunculosis Committee, HMS Stationary Office, Edinburgh, 1935.

7. Schäperclaus, W., *Fishkrankheiten. Akedemie-Verlag.,* Berlin, 1954, 3rd ed., 537 pp.

Received March 27, 1956. P.S.E.B.M., 1956, v92.

Role of Thioctic Acid in Chick Nutrition. (22393)

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Thioctic acid (6, 8-dithiooctanoic acid) is known to be essential for the nutrition of several microorganisms and to be a component of keto acid oxidases. Evidence regarding its role in nutrition has been conflicting. Stokstad *et al.*(1) were unable to obtain any evidence of a thioctic acid deficiency in either chicks or rats on purified type diets containing alcohol extracted casein. DeBusk and Williams(2), however, reported that thioctic acid increased growth of chicks on a purified type diet and that 1 mg per kg per day was required to give maximum response. This communication deals with additional experiments on the dietary requirements of chicks for thioctic acid and on the synthesis of thioctic acid by the developing chick embryo.

Methods. Day-old Barred Rock x New Hampshire cross bred chicks were kept in thermostatically-controlled heated battery brooders and given feed and water *ad libitum*. Diets are shown in Table I. Casein was extracted continuously with hot ethanol for 20 hours. Thioctic acid was assayed by the pad plate procedure using *Corynebacterium bovis* (3). Samples were prepared for thioctic acid assay by grinding 5 g fresh tissue with 10 ml of 9 N H_2SO_4 in a tissue homogenizer, autoclaving for 3 hours at 120°C, neutralizing and diluting to 50 ml. Insoluble material was

TABLE I. Composition of Diets.

	Diet 1	Diet 2	Diet 3
Casein (alcohol extracted)	—	20	—
Soybean protein*	—	—	22
" meal	35	—	—
Sucrose	—	60	66
Ground yellow corn	59	—	—
Gelatine	—	8	—
Methionine	.3	—	—
Cystine	—	.4	—
Glycine	—	—	.3
Calcium gluconate	—	5	—
Bone ash	—	2	2.5
Dicalcium phosphate	2.5	—	—
Calcium carbonate	1.0	—	1.0
Salt mixture†	.7	1.4	1.4
Sodium chloride	.8	.6	.6
Vit. mixture in glucose‡	1	1	1
Corn oil + vit. A, D and E§	—	1	5
Choline chloride	—	.2	.2
Dry vit. A + D‖	.1	—	—
Vit. E acetate supplement	.040	—	—

*"Sodium proteinate" (Archer Daniels Midland Co.).

† Salt mixture had the following composition: K_2HPO_4 300, KH_2PO_4 225, $MgSO_4$ 125, $MnSO_4$ (anhydrous) 20, ferric citrate 25, $CuSO_4 \cdot 5H_2O$ 1.0, KI 0.3, zinc acetate 0.7, $Al_2SO_4 \cdot 18H_2O$ 0.8, cobalt acetate 0.2, nickel carbonate 0.1. (Total 715.)

‡ Vitamin mixture supplied the following/kg of diet: thiamine 10 mg, riboflavin 10 mg, pyridoxine 10 mg, niacinamide 50 mg, calcium pantothenate 50 mg, folic acid 2 mg, biotin 0.2 mg, 1-acetoxy-2-methyl-4-napthyl sodium phosphate (vit. K derivative) 5 mg.

§ Vit. A acetate 15,000 U.S.P. units, vit. D_3 2,000 A.O.A.C. units, mixed tocopherols 340 mg/kg diet.

‖ Vit. A 10,000; vit. D_3 2,000.

removed by filtering; 0.01 to 0.02 ml of this solution was added to ¼" filter paper discs which were then placed on an agar plate seeded with *C. bovis*. The standard consisted of several discs containing from 0.3 to 100 mμg of dl-thioctic acid. The zones were read after 16 to 20 hours incubation at 37°C. Thioctic acid was synthesized by Dr. M. W. Bullock using the procedure described elsewhere(4).

Results. The effect of thioctic acid on chick growth was determined on a purified type diet containing hot alcohol-extracted casein and on a diet containing corn and soybean meal. Thioctic acid at levels of 1.0 or 10 mg per kg of diet did not increase growth of chicks on either of these diets (Table II). Similar results were obtained in other experiments with the casein diet. Thioctic acid was also fed to chicks raised in a brooder house under commercial rearing conditions. The diet was the practical type corn-soybean diet (diet 1) modified by inclusion of 0.025% nitrophenide to control coccidiosis. The results presented in Table III show that thioctic acid at levels of 1 and 5 mg per kg of diet was without effect on growth. In order to determine whether differences in the diets were responsible for the discrepancy between the results of DeBusk and Williams and our own, we obtained a sample of their diet but were unable to obtain any growth response to thioctic acid on it. In this experiment in which duplicate groups were used, unsupplemented chicks averaged 109 g at 12 days and those receiving the same diet supplemented with 5 ppm dl-thioctic acid averaged 103 g.

TABLE II. Effect of Thioctic Acid on Growth of Chicks. 12 chicks/group.

Type of diet	Thioctic acid, mg/kg diet	Body wt at 28 days (g)	Avg (g)
Corn, soybean (Diet 1)	0	318 346	332
Idem	1	348 346	347
Sucrose, casein (Diet 2)	0	259 277	268
Idem	1	244 252	248
"	10	253 262	258

Since it appears that under our experimental conditions chicks did not require an exogenous source of thioctic acid, the question arises whether thioctic acid is formed by intestinal synthesis or within the animal tis-

TABLE III. Effect of Thioctic Acid on Growth of Chicks on a Practical Broiler Ration.

Ration No. 1	300 chicks/group	
	Avg wt at 10 wk	
Supplement/kg diet	g	Avg
0	1290 1372 1280	1314
1 mg dl-thioctic acid	1314 1311 1312	1312
5 mg " "	1321 1340 1290	1317

TABLE IV. Thioctic Acid Content of Developing Chick Embryo.

Age (days)	Embryo		Non-embryonic part of egg		Total thioctic acid (γ/egg)*
	Wt (g)	Thioctic acid (γ/g)*	Wt (g)	Thioctic acid (γ/g)*	
0	—	—	49	.00	.5
9	1.6	.07	48	.02	.8
12	5.1	.12	44	.02	1.3
15	12.5	.09	37	.03	2.3
19	30	.24	18	.07	8.9
21	41	.28	—	.00	11.5

* Expressed as 0.5 value obtained/g wet tissue by using DL-thioctic acid as standard, since the L-isomer is inactive in the microbiological assay (10).

sues. This was studied by assaying the thioctic acid content of the developing chick embryo. The thioctic acid content of fresh eggs and embryos taken at varying ages is shown in Table IV. Each value represents the average of 2 separate experiments in which 3 eggs were pooled to form an assay sample. Fresh eggs contained a total of less than 0.5 γ of thioctic acid and 21-day embryos contained a total of 11.5 γ. The thioctic acid formed during development was largely found in the embryo rather than in the "yolk" or non-embryonic part of the developing egg. It is apparent that the chick embryo is able to synthesize thioctic acid. Olson and Dinning(5) have reported that a deficiency of sulfur amino acids produced a reduction in pyruvic acid oxidation by liver tissue and a reduction in coenzyme A levels. Since thioctic acid is a part of the pyruvic acid oxidation system it was of interest to determine whether thioctic acid levels are also affected by a sulfur amino acid deficiency. Chicks were raised on a methionine-deficient diet containing purified soybean protein (diet 3). The tissue levels of thioctic acid in chicks raised on this diet with and without methionine are shown in Table V. It is apparent that although the diet was sufficiently deficient in methionine to severely restrict growth, there was no effect on the thioctic acid level in the heart and liver.

TABLE V. Effect of Methionine Deficiency on Thioctic Acid Content of Chick Tissue.

Age (days)	Body wt		Liver		Heart	
	No meth. (g)	3 g meth. (g)†	No meth. (γ/g)*	3 g meth. (γ/g)*	No meth. (γ/g)*	3 g meth. (γ/g)*
0	41	41	2.0	2.0	.6	.6
7	54	68	1.4	1.2	.8	.6
15	66	96	1.5	1.8	.7	1.1
21	58	133	1.5	1.5	.8	.6

* Expressed as 0.5 value obtained using DL-thioctic acid as standard.
† Per kilo of diet.

Discussion. The addition of thioctic acid to a purified diet containing casein as a protein source did not stimulate the growth of chicks. It is difficult to reconcile these data with those of DeBusk and Williams in which marked growth responses were obtained. This discrepancy is not due to differences in the diets employed at the two laboratories as a sample of diet obtained from the Texas workers gave a result similar to that on our own diet. It appears that the only difference between our experimental conditions and those of DeBusk and Williams is the source of the chicks. These workers also reported that chicks on a commercial broiler mash responded to thioctic acid while in our own laboratory no response was obtained with diet 2 which is a practical type of diet containing corn and soybean meal. Similar negative results with thioctic acid have been observed by Combs(6), Norris(7), and Supplee, *et al.*(8).

The finding that thioctic acid is synthesized by the developing chick embryo makes it probable that the growing chick is also able to synthesize thioctic acid. It does not, however, prove that an exogenous source of thioctic acid may not be needed by the chick. This is the case with nicotinic acid, which is required in the diet of the growing chick unless unusually large quantities of tryptophan are supplied, but which is also synthesized by the developing chick embryo(9).

Summary. 1. Thioctic acid did not increase growth in chicks on purified type diets containing casein and glucose, or on a diet of natural foodstuffs. 2. The developing chick embryo synthesized thioctic acid. 3. A methionine deficiency in the chick did not decrease the level of thioctic acid in liver and heart tissues.

1. Stokstad, E. L. R., Broquist, H. P., and Patter-

son, E. L., *Fed. Proc.*, 1953, v12, 430.

2. DeBusk, B. G., and Williams, R. J., *Arch. Biochem.*, 1955, v55, 587.

3. Stokstad, E. L. R., Seaman, G. R., Davis, R. J., and Hutner, S. H., *Vitamin Assay Methods*, in press.

4. Bullock, M. W., Brockman, J. A., Jr., Patterson, E. L., Pierce, J. V., von Saltza, M. H., Sanders, F., and Stokstad, E. L. R., *J. Am. Chem. Soc.*, 1954, v76, 1828.

5. Olson, R. E., and Dinning, J. S., *Proc. N. Y. Acad. Sci.*, 1954, v57, 889.

6. Combs, G. F., personal communication, 1955.

7. Norris, L. E., personal communication, 1955.

8. Supplee, *et al.*, *Arch. Biochem. Biophy.*, 1956, v61, 140.

9. Snell, E. E., and Quarles, E., *J. Nutrition*, 1941, v22, 483.

10. Walton, E., Wagner, A. F., Bachelor, F. W., Peterson, L. H., Holly, F. W., and Folkers, K., *J. Am. Chem. Soc.*, 1955, v77, 5144.

Received March 29, 1956. P.S.E.B.M., 1956, v92.

Antibodies to APC Virus Type 8 in Epidemic Keratoconjunctivitis.* (22394)

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Epidemic keratoconjunctivitis (EKC) is an infectious eye disease with well-defined clinical characteristics. After an incubation period of 7 to 10 days there develops an intense follicular conjunctivitis, sometimes with pseudomembrane formation, accompanied by enlarged tender preauricular lymph nodes. In most cases 5 to 10 days later corneal lesions develop. These are characteristically small, round, subepithelial corneal opacities without superficial ulceration or impaired sensitivity of the cornea. These opacities may persist up to 2 years and may seriously interfere with vision. The disease has occurred in large epidemics in Europe, the Far East, Hawaii, and the Pacific Coast of the United States. Smaller outbreaks are seen from time to time all over the world.

It is generally believed that EKC is of viral etiology and many claims have been made for the isolation of a specific causative agent. These were reviewed by Cockburn(1) who concluded that "there is at present no virus available that can be regarded with confidence as the etiologic agent of EKC." In the course of studies on various types of keratoconjunctivitis we isolated a virus from EKC (2) which has been designated the prototype of APC virus type 8(3). Initial serological observations showed a high incidence of neutralizing antibodies to that virus in patients with EKC and a virtual absence of such antibodies in other individuals(4). Consequently, a comprehensive serological survey was undertaken to explore this association. The present paper summarizes the results obtained to date with sera from several widely separated areas of the world.

Materials and methods. Viruses. The virus isolated in tissue culture from a patient with typical EKC ("TRIM") was employed in the 4th to 14th passage in the form of supernatant fluid from infected HeLa cell cultures. This virus (APC type 8) was propagated routinely by inoculating twice-washed HeLa cell cultures obtained from Tuskegee Institute, Ala., with 0.1 ml of undiluted stock virus, adding 0.9 ml of maintenance medium† (10% chick serum in mixture 199 containing Penicillin 500 *u*/ml, Streptomycin 500 μg/ml, and Rimocidin 10 μg/ml) and incubating the tubes at 36°C for 2-4 days, until cytopathogenic effects had progressed to 3+ or 4+. The fluid from individual tubes (containing de-

* Supported by grants from the National Institutes of Health (B604), Burroughs Wellcome and Co., and Committee on Research, University of California School of Medicine.

† All tissue culture media were obtained from Microbiological Associates, Bethesda, Md.

generated and detached cells) was pooled and kept at –20°C until used in neutralization tests. The infective titer of pools never exceeded 10^{-2}, was not diminished by prolonged storage, and was not increased by disrupting the cells with repeated freezing and thawing (2). The "E.K." strain of St. Louis encephalitis virus was kindly supplied in the form of 8th passage mouse brain by Dr. W. McD. Hammon. It was passed intracerebrally in 21 day Swiss mice and a virus pool consisting of a 20% brain suspension in skim milk was kept at –40°C until used in mouse neutralization tests. *Sera.* Whenever possible paired or serial serum specimens were secured from patients seen during acute EKC. However, in many instances only single sera were available which had been obtained from 2 weeks to 8 years after onset of EKC. To estimate the frequency of antibodies to APC virus type 8 in the general population and in other eye diseases, sera were obtained from a variety of individuals from the same geographic areas and age distribution represented by the EKC patients. We are greatly indebted to Dr. H. L. Ormsby for serum specimens from Canada (Windsor 1951 and Toronto 1953-55), to Dr. M. D. Pearlman for sera from Chicago (1954), to Dr. I. Leopold for sera from Philadelphia collected in 1955 after an outbreak in 1953, and to Doctors H. Konig and R. Witmer for sera from Switzerland (1955). Sera from EKC patients and normals from the Como area of Italy (1955) were kindly supplied by Prof. G. Bietti, and from the Kumamoto area (Kyushu) of Japan (1955) by Prof. Y. Mitsui. Most other sera were secured from patients seen by ophthalmologists in California for either EKC or unrelated eye diseases, and from persons without any eye disease. All sera were stored at –20°C, and were inactivated by heating at 56°C for 30 minutes before use in neutralization tests. *Neutralization tests.* These were performed as previously described(2,4). Briefly, the technic consisted in mixing equal volumes of inactivated serum dilution (in mixture 199) and undiluted virus pool, and incubating the mixture for 30 minutes at room temperature. The mixture (0.2 ml) was then inoculated into twice-washed HeLa cell cultures and 0.8 ml maintenance medium was added. The tubes were incubated at 36°C in a stationary position without change of medium and inspected daily for cytopathogenic effects. Readings were taken for at least 4 days after degeneration of the control tubes (virus plus normal rabbit serum) was complete. Tubes were read at random and cytopathogenic changes were graded in the conventional fashion (0 to 4+). Agreement between tubes inoculated with the same mixture was good. The results were taken to indicate neutralization only when there was a difference of 3+ in readings of experimental and control tubes for at least 2 consecutive days. The results are expressed as the final dilution of serum in the serum-virus mixture which prevented cytopathogenic effects. Some difficulty was encountered because of the very low titers of the available virus pools (the greatest amount of virus per tube being 50 TC50), and because of some variability in the susceptibilities of different lots of HeLa cells. Certain animal sera delayed cytopathogenic effects, even though they contained no antibody, and could therefore not be employed. It was necessary to include in every neutralization test positive control sera of known antibody content, negative control sera lacking antibody, and virus titrations. The results were accepted for inclusion in this report only when all these controls were satisfactory. The titers of virtually all positive human sera were determined more than once. Repeated determinations of antibody in a given serum specimen generally agreed within one 2-fold dilution of serum. Paired serum specimens were always titrated simultaneously. Mouse neutralization tests with the "E.K." strain of St. Louis encephalitis were performed in the conventional manner, using inactivated undiluted serum and tenfold virus dilutions.

Results. Frequency of APC 8 neutralizing antibodies. The present series includes 70 cases of EKC and 140 controls. Of the EKC patients 60 had the typical disease, whereas in 10 others, some feature of the complete picture was lacking, most often keratitis or corneal opacities. The serological results are summarized in Table I. It is seen that the vast majority of EKC patients had neutral-

TABLE I. Incidence of Neutralizing Antibodies to APC 8 Virus in EKC Patients and Controls.

Sera from	No. sera	Neutralizing antibodies in 1:10 dilution: Present No.	Present %	Absent No.	Absent %
EKC					
U.S.-Canada	30	29	96.7	1	3.3
Switzerland	15	13	86.7	2	13.3
Italy	13	13	100.0	0	.0
Japan	12	11	91.7	1	8.3
EKC–Total	70	66	94.3	4	5.7
Controls					
U.S.-Canada	104	5	4.8	99	95.2
Italy	24	1	4.2	23	95.8
Japan	12	4	33.3	8	66.7
Controls–Total	140	10	7.1	130	92.9

izing antibodies in a serum dilution of 1:10 or greater. This was the case in 96.7% of cases of typical EKC and 80% of atypical disease. Such antibodies occurred infrequently in controls including normal individuals, patients with urinary tract infections, skin herpes, or a variety of eye diseases such as herpetic keratitis, uveitis, and several types of conjunctivitis. Because of the greater frequency of EKC in Europe and Japan, it was important to examine sera from normal individuals residing in the same area as the EKC patients. Whereas antibodies to APC 8 in the absence of clinical EKC were rare in North America and Italy, they occurred in 4 of 12 individuals chosen at random in the Kumamoto area of Japan. In that region clinical EKC occurs at a high endemic level at the present time.

Sera from EKC and controls were well matched in age distribution, covering a span of from 10 to 75 years. EKC may occur at all ages but typical cases are uncommon in children(5). The Italian EKC patients were from 18 to 70 years old, and among 24 controls ranging from 11 to 75 years, only one person (34 years old) had an antibody titer of 1:10, and 2 others aged 20 and 47 had a titer of 1:5. The Japanese EKC patients ranged from 11 to 64 years and the controls from 14 to 66 years. The single Japanese EKC patient without APC 8 antibodies was 14 years old, whereas the 4 controls with APC 8 antibodies were 14, 23, 49, and 51 years old. The North American EKC patients as well as controls are weighted toward middle age. The low incidence of APC 8 antibodies in the general population in the Western United States and Canada (Table I) was paralleled by a survey of the clinical population in Washington, D.C.(6). There APC 8 antibodies in a serum dilution of 1:8 were found in 1 of 44 sera (2.2%) from the ages 6 to 15, and in 3 of 46 sera (6.5%) from the ages 31 to 77. The comparable age distribution of EKC cases and controls indicates that the observed differences in the incidence of APC 8 antibodies are not due to errors in sampling.

Titer of APC 8 neutralizing antibodies. The distribution of neutralizing antibody titers is shown in Table II. Among 60 patients with typical EKC 62% had a titer of 1:40 or greater and 87% had a titer of 1:20 or greater. Similarly, 8 of 10 cases of atypical EKC had a titer of 1:20 or greater. Of 140 controls only 3 (2.2%) had a titer of 1:20 or greater. Some of the EKC patients included in Table II contracted the disease months or even years before the serum was obtained. This circumstance may have influenced the distribution of antibody titers, because antibody levels decline relatively soon after infection. In Table III antibody titers are arranged according to the interval between onset of EKC and the date of securing the serum specimen. In spite of the small number of patients in some groups the trend is evident. While more than 70% of patients bled within 6 months of onset had antibody titers of 1:40 or greater, this occurred in only

TABLE II. Titer of Neutralizing Antibodies to APC 8 Virus in EKC Patients and Controls.

Serum from	No. sera	Neutralizing antibodies present in a serum dilution of 1 to: <10	10	20	40	80	>80
EKC							
U.S.-Canada	30	1	6	9	5	7	2
Switzerland	15	2	0	2	7	3	1
Italy	13	0	0	5	4	4	0
Japan	12	1	0	2	3	5	1
EKC–Total	70	4	6	18	19	19	4
Controls							
U.S.-Canada	104	99	3	2	0	0	0
Italy	24	23	1	0	0	0	0
Japan	12	8	3	0	1	0	0
Controls–Total	140	130	7	2	1	0	0

20% of those bled 1-2 years after onset, and in none of those bled more than 2 years after onset. Thus it is apparent that the search for APC 8 antibodies could not be applied reasonably to patients who suffered EKC infection prior to 1951 unless their sera had been preserved from the time of active disease.

Rise in titer of neutralizing antibodies to APC 8 during acute EKC. Paired sera were secured from 18 individuals whose initial serum specimen had been obtained before the 14th day after onset of EKC. Seventeen of them reached neutralizing antibody titers of 1:10 or greater at some time during their illness. The remaining one (a Japanese boy aged 14) never developed either keratitis or measurable APC 8 antibodies. Fifteen patients had a 4-fold or greater rise in APC 8 antibodies during their illness (examples in Table IV). The speed and magnitude of titer rise and fall varied greatly. One woman had a peak titer of 1:40 in her initial serum obtained on the 13th day after onset of EKC, showed no further rise, and her titer fell to 1:20 by the 60th day. The majority of patients from whom serial serum specimens were available reached peak titer (1:40 to 1:160) in 3 to 6 weeks after onset of EKC, and maintained it for some weeks. The highest APC 8 antibody titer was 1:640 in a Japanese with severe EKC. We observed one patient who reached a maximum titer of 1:80 on the 44th day after onset, maintained it through the 80th day, but gradually declined to 1:20 by the 380th day. That sequence may well occur in many individuals after EKC infection, and may account for the low titer of APC 8 antibodies in patients bled more than 6 months after the onset of EKC.

TABLE III. Presence of Neutralizing Antibodies to APC 8 Virus at Intervals after Onset of Acute EKC.

Time after onset of EKC	No. patients	Neutralizing antibodies present in a serum dilution of 1 to			
		<10	10	20	40 or greater
<6 mo	56	3	1	12	40
1-2 yr	10	1	3	4	2
2-4 "	4	0	2	2	0
8 "	5*	2	2	1	0

* These 5 patients are not included on the present series of 70 patients with EKC.

TABLE IV. Homotypic and Heterotypic Antibody Titers in Patients with EKC Infections.

Location	Patient	Days after onset	Titer of antibodies to APC 8	Titer of antibodies to APC 3
Japan	Hira	3	10*	40
		24	320	40
		31	640	ND
"	Naga	3	10	80
		24	40	ND
		31	80	80
"	Kota	13	10	5
		20	40	ND
		37	80	10
Chicago	Quar	4	<10	<10
		33	80	<20
"	Lars	3	<10	10
		19	40	20
"	Hans	2	<10	40
		24	80	40
Calif.	Cott	6	<10	20
		80	80	40

* Reciprocal of highest final serum dilution neutralizing approximately 50 TC_{50} of virus.
ND = Not done.

Antibodies to other APC types in EKC. In view of the antigenic relationships which exist within the group of APC viruses, heterotypic antibody rises must be considered. APC type 3 is a common cause of external eye infection. Therefore, it seemed desirable to investigate possible serological cross reactions between APC types 3 and 8. In Table IV are listed the APC 8 antibody titer changes in a few representative patients with EKC. The rise in APC 8 antibody titer was not accompanied by a heterotypic rise in APC 3 antibody titer. We tested 10 serum pairs from individuals with infections due to APC types 2, 3, and 6. There was a significant homotypic antibody titer rise, but no rise to APC 8. Thus it appears that the rise in neutralizing antibody titer to APC 8 is not due to infection by other known APC types, but most likely is caused by APC 8 infection.

Antibodies to the preserved "E.K." virus or St. Louis encephalitis virus. A virus prominently associated with EKC in the literature was isolated by Sanders(7). At present its nature and whereabouts are uncertain and the original agent may have been lost. The strains labeled "E.K." virus and preserved in

several laboratories, and perhaps derived from Sanders' isolation, are immunologically closely related to St. Louis encephalitis(8). We tested 15 sera from patients with typical EKC against this virus by mouse neutralization test. All these sera contained antibodies against APC 8 virus. None of them neutralized the "E.K." virus to any significant extent. Thus there is no serological evidence that the virus labeled "E.K." is associated with the disease EKC as it has occurred since 1951(1).

Discussion. The serological data presented in this paper reveal a striking and unequivocal association between epidemic keratoconjunctivitis and neutralizing antibody to APC virus type 8. This antibody can be presumed to have arisen as a result of infection with APC virus type 8, or an antigenically closely related agent. The rise in antibody titer which occurs during the acute phase of EKC and the gradual loss of titer thereafter are in agreement with this concept. The low incidence of APC 8 antibodies in the general population in America and Italy contrasts with the more frequent occurrence of such antibodies in individuals without EKC in Japan. In Japan EKC occurs at a high endemic level and subclinical infections are to be expected, whereas in America and Italy, the disease is only seen in occasional circumscribed epidemics. All these features support the concept that infection with APC virus type 8 (or an agent closely related antigenically) is regularly associated with clinical EKC. This statement applies only to patients infected with EKC between 1951 and 1955. Because of the rapid fall in APC 8 antibody titer within a year of onset of EKC, one cannot expect to find with any regularity significant antibody titers to APC 8 in individuals who contracted EKC prior to 1951. Thus it is not possible to state whether APC 8 infection was associated with EKC prior to that time, unless sera collected within one year of onset of EKC could be tested. Unfortunately, we have been unable as yet to secure any such specimens.

The outbreaks of EKC in America prior to 1950 have been generally attributed to the agent described by Sanders(7). The presently available strains of "E.K." virus—possibly derived from Sanders' agent—are not neutralized by sera from patients who contracted EKC since 1951(1,8). Thus it is evident that typical EKC occurring between 1951 and 1955 in Italy, Switzerland, America, and Japan were not associated with infection by that agent.

The present serological study strongly suggests that APC 8 virus (or an antigenically related agent) is involved in the production of the clinical disease EKC. In order to establish its etiologic role it would be necessary to isolate the virus regularly from the disease and to reproduce the typical illness by experimental inoculations of human volunteers. Such studies are currently under way.

Summary. The sera from patients with epidemic keratoconjunctivitis (EKC) in Japan, Italy, Switzerland, and North America regularly contain neutralizing antibodies to APC virus type 8, whereas such antibodies are absent from the general population in these areas. Paired sera from patients with EKC show a significant rise in neutralizing antibodies to APC virus type 8. These antibodies persist for only a limited period after the onset of the disease. The constant association of clinical EKC and antibodies to APC virus type 8 suggests that this agent (or antigenically related virus) may play a role in the etiology of this disease.

1. Cockburn, T. A., *Am. J. Ophthal.*, 1954, v38, 476.

2. Jawetz, E., Kimura, S. J., Hanna, L., Coleman, V. R., Thygeson, P., and Nicholas, A., *ibid.*, 1955, v40, 200.

3. Huebner, R. J., Bell, J. A., Rowe, W. P., Ward, T. G., Suskind, R. G., Hartley, J. W., and Pfaffenbarger, R. S., *J. Am. Med. Assn.*, 1955, v159, 986.

4. Jawetz, E., Kimura, S., Nicholas, A., Thygeson, P., and Hanna, L., *Science*, 1955, v122, 1190.

5. Mitsui, Y., Tanaka, C., and Yamashita, K., *Am. J. Ophthal.*, 1955, v39, 540.

6. Rowe, W. P., Huebner, R. J., personal communication.

7. Sanders, M., and Alexander, R. C., *J. Exp. Med.*, 1943, v77, 71.

8. Cheever, F. S., Proc. Soc. Exp. Biol. and Med., 1951, v77, 125.

Received April 3, 1956. P.S.E.B.M., 1956, v92.

Specific Determination of Hydroxy-L-Proline in Biological Materials.* (22395)

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Paper chromatographic studies on the amino acid metabolism of animal tissues *in vitro*(1) have shown that marked changes in amino acid content of synthetic medium M 150(2,3) occur during the cultivation period. Medium M 150 contains 60 ingredients, including 20 amino acids, in a modified Tyrode's solution(4) and its complexity made it difficult to detect changes in amino acids that were present in low concentrations or that did not separate completely on the chromatograms. Particular difficulty was encountered with hydroxyproline, which reacted only slightly on development with ninhydrin(5), isatin(6), vanillin(7) or isatin and *p*-dimethylaminobenzaldehyde(8). For this reason, an effort was made to develop a selective method for hydroxyproline that could be applied in the presence of high concentrations of other amino acids. The present communication reports such a specific method, based on a modification of the conventional ninhydrin procedure. The application of this method to complex synthetic media and to deproteinized chick embryo extract is presented.

Methods. Samples of media for analysis (5.0 ml) were concentrated to dryness *in vacuo* over H_2SO_4, reconstituted in 0.2 ml of deionized water and 10 λ portions used without desalting. One-dimensional descending chromatograms (Schleicher and Schuell No. 597 or Whatman No. 1 paper) were developed for 2 successive 18-hour periods in either *n*-butanol-acetic acid-water or *n*-butanol-ethanol-water. The chromatograms were dried at 110°C for 2 to 3 minutes and sprayed with 0.4% ninhydrin in water-saturated *n*-butanol or 95% ethanol. Full details of the chromatographic procedures have been reported previously(1,9,10). Chick embryo extract was prepared by grinding 11-day-old embryos and centrifuging off the tissue pulp(11). The supernatant extract was adjusted to pH 4.0 with 1 N H_2SO_4, precipitated proteins removed by centrifuging, and the pH readjusted to 7.2. Two volumes of acetone were added, the mixture allowed to stand overnight in the refrigerator, and centrifuged. The final supernatant was concentrated to dryness in the usual manner and used for analysis. Synthetic medium M 150 was prepared as described previously(2,3). All chemicals and reagents were of the highest purity obtainable. Spectrophotometric measurements were made by cutting appropriate areas from the developed chromatograms and fixing them to the inner walls of 1 cm Corex cells. Determinations were then made in a Beckman, Model DU. Ultraviolet examinations were carried out with a laboratory hand lamp (short wave model, 2537 Å).

Results. Development of a specific color with hydroxy-L-proline and ninhydrin. Previous studies on the determination of phenylalanine in synthetic medium M 150(10) showed that dipping ninhydrin-developed chromatograms in 1% sodium bicarbonate produced a stable blue color characteristic of this amino acid. At the same time, this alkali treatment converted the faint yellow hydroxyproline chromogen to a bright pink spot that faded within 5 minutes. Further studies on this reaction have now shown that dipping the chromatograms into 1 N HCl, within 2 minutes of the bicarbonate treatment, intensifies and stabilizes the pink hydroxyproline chromogen, and discharges the characteristic phenylalanine color. The hydroxyproline chromogen was firmly bound to the paper chromatograms and could not be eluted with water. Washing of the chromatograms with water removed the majority of other ninhydrin and amino acid colors and left the hydroxyproline chromogen as a clear and dis-

* The cooperation of Mr. C. E. Kerr, of this Department, in making the photographic preparations, is gratefully acknowledged.

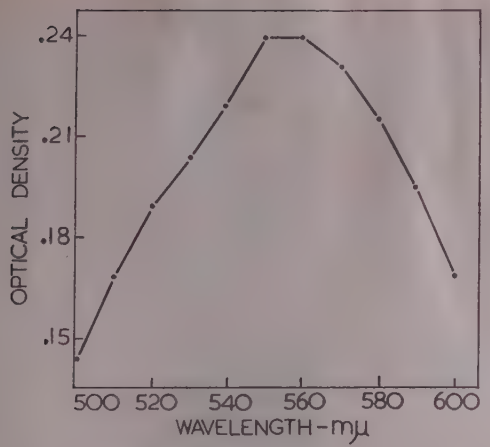

FIG. 1. Absorption curve of new hydroxyproline chromogen. Measurements made in Beckman, Model DU, using 1 cm Corex cells containing area cut from developed chromatogram. 25 μg hydroxy-L-proline.

tinct spot. The absorption curve of this new hydroxyproline color is presented in Fig. 1 and shows an absorption maximum at 550 to 560 mμ. It is evident that the alkali and acid treatment has caused a major shift in the absorption maximum away from the characteristic yellow imino acid and ninhydrin color (absorption maximum at 440 mμ).

Application to synthetic media and deproteinized embryo extract. The application of the method to synthetic medium M 150 containing different levels of hydroxyproline and proline and to deproteinized chick embryo extract is illustrated in Fig. 2. This figure shows 2 identical chromatograms, both developed by the conventional ninhydrin procedure but the second subsequently treated with bicarbonate and hydrochloric acid. By the conventional ninhydrin method (Fig. 2, 1 to 6), the presence of hydroxyproline in these materials is almost impossible to detect, due to the faintness of the yellow color produced and overlapping by more strongly-reactive amino acids. Application of the new procedure (Fig. 2, 1_4 to 6_4) removes the interfering amino acids, and hydroxyproline can now be detected readily by its characteristic color. As little as 5 to 10 μg of hydroxyproline can be demonstrated, either alone or in synthetic medium M 150. At the same time, the failure of proline to react increases the specificity of the method. The chromatogram also shows (Fig. 2, 3_4) that hydroxyproline is one of the major components of deproteinized chick embryo extract.

Fluorescence under ultraviolet light. Examination of the chromatograms under a hand ultraviolet lamp showed that the new hydroxyproline chromogen exhibited a strong pink fluorescence. This property could not be detected after the conventional ninhydrin development nor after subsequent alkali treatment but appeared immediately on acid and water treatment. In Fig. 3 is shown the hydroxyproline region of the previous chromatogram (Fig. 2) photographed under ultraviolet light. The marked fluorescence of the hydroxyproline chromogen is clearly shown in the chromatogram developed by the present procedure while only a faint fluorescence is visible in the areas developed by the conventional ninhydrin method. As little as 5 μg of hydroxyproline exhibits strong fluorescence (Fig. 2, 6_4). In additional experiments 1 to 5 μg were detected readily.

Discussion. Studies on the hydroxyproline content of foods(12) and biological materials generally have been handicapped by the lack of a specific method that can be applied to the analysis of complex mixtures and by the unavailability of a microbiological method for this amino acid(13). The majority of the methods so far devised either fail to distinguish clearly between hydroxyproline and proline or cannot be applied to materials in which the hydroxyproline concentration is low in proportion to that of other amino acids. The present method overcomes these difficulties through the formation of a characteristic chromogen specific for hydroxyproline but not proline. Moreover, the method was found suitable for the analysis of synthetic media in which hydroxyproline represented only 1% of the total amino acids present. Because the specific hydroxyproline chromogen remained fast to the paper chromatograms, it became possible to wash away, with water, overlapping spots and determine this amino acid under conditions of poor separation. In this way, deproteinized chick em-

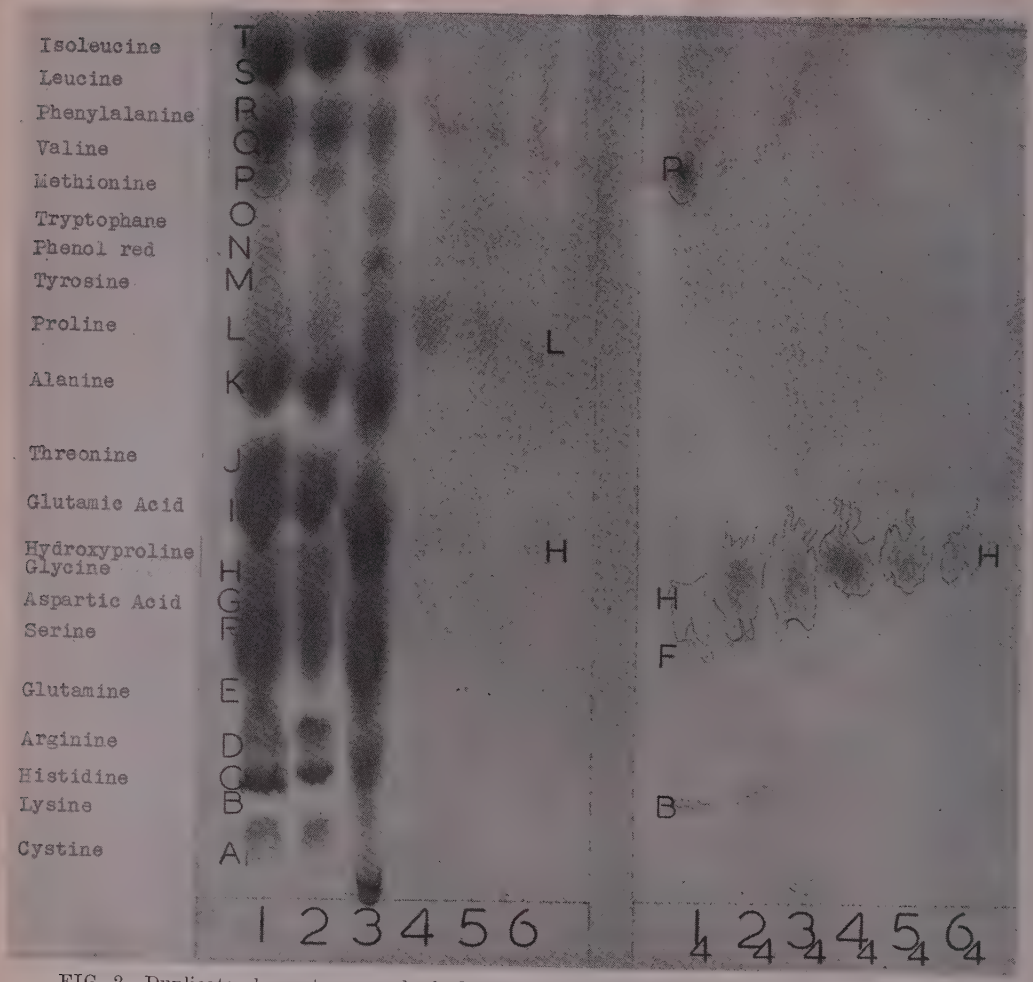

FIG. 2. Duplicate chromatograms, both developed by ninhydrin, but chromatogram on right subsequently treated with 1 N $NaHCO_3$, 1 N HCl and washed with water. 1 and 1_4, synthetic medium M 150, containing 5 μg of hydroxyproline and 20 μg of L-proline; 2 and 2_4, M 150 containing 25 μg of hydroxyproline and proline; 3 and 3_4 deproteinized chick embryo extract; 4 and 4_4, 25 μg of hydroxyproline and proline in water; 5 and 5_4, 10 μg of hydroxyproline and proline in water; 6 and 6_4, 5 μg of hydroxyproline and proline in water.

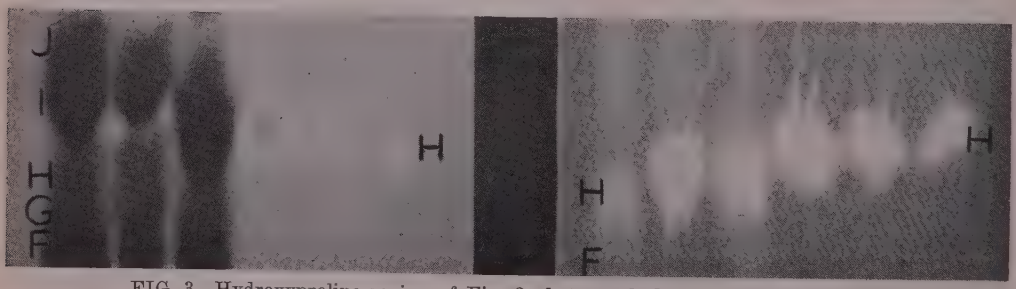

FIG. 3. Hydroxyproline region of Fig. 2 photographed under ultraviolet light.

bryo extract was found to contain a relatively high concentration of hydroxyproline. This observation is in marked contrast to the low level reported by Westfall, Peppers and Earle (14), who used the conventional isatin method.

This procedure for hydroxyproline has been used successfully in studies on the amino acid metabolism of tissues cultivated in synthetic medium M 150(1). This precludes interference by approximately 60 compounds of biological importance, including amino acids, vitamins, purines and pyrimidines, salts and certain accessory growth factors. The only compound observed to form a somewhat similar compound was phenylalanine but this chromogen was discharged by the acid treatment used to stabilize the hydroxyproline color. An unusual feature of the method is that it can be applied to paper chromatograms previously developed with ninhydrin for the measurement of other amino acids. The chemical nature of the hydroxyproline chromogen has not been determined, although there appears to be some resemblance to the benzene-soluble red compound observed by Troll and Cannan(15). It should be noted, however, that the chromogen isolated by these workers was formed by proline as well as by hydroxyproline. The intensity of the specific chromogen and its fluorescence varied with the concentration of hydroxyproline and attempts to develop a quantitative method are now in progress.

Summary. A new, specific method for hydroxyproline is reported, based on the development of a characteristic chromogen through ninhydrin development of paper chromatograms followed by rapid treatment with dilute alkali and dilute acid. The new chromogen has an absorption maximum at 550 to 560 mμ and fluoresces strongly under ultraviolet light. Application of the method to complex biological media showed that 5 to 10 μg of hydroxyproline could be detected. A high level of this amino acid was shown in chick embryo extract for the first time.

1. Pasieka, A. E., Morton, H. J., and Morgan, J. F., *J. Nat. Cancer Inst.*, 1956, v16, 995.

2. Morgan, J. F., Morton, H. J., and Parker, R. C., PROC. SOC. EXP. BIOL. AND MED., 1950, v73, 1.

3. Morgan, J. F., Campbell, M. E., and Morton, H. J., *J. Nat. Cancer Inst.*, 1955, v16, 557.

4. Hanks, J. H., and Wallace, R. E., PROC. SOC. EXP. BIOL. AND MED., 1949, v71, 196.

5. Dent, C. E., *Biochem. J.*, 1948, v43, 169.

6. Acher, R., Fromageot, C., and Jutisz, M., *Biochim. et Biophys. Acta*, 1950, v5, 81.

7. Jepson, J. B., and Smith, I., *Nature*, 1953, v172, 1100.

8. Curzon, G., and Giltrow, J., *ibid.*, 1954, v173, 314.

9. Pasieka, A. E., and Morgan, J. F., *Biochim. et Biophys. Acta*, 1955, v18, 236.

10. ———, *ibid.*, 1956, v19, 356.

11. Morgan, J. F., and Parker, R. C., PROC. SOC. EXP. BIOL. AND MED., 1949, v71, 665.

12. Aronson, J. R., and Elvehjem, C. A., *Food Res.*, 1956. v21, 109.

13. Dunn, M. S., Shankman, S., Camien, M. N., Frankl, W., and Rockland, L. B., *J. Biol. Chem.*, 1944, v156, 703.

14. Westfall, B. B., Peppers, E. V., and Earle, W. R., *J. Nat. Cancer Inst.*, 1954, v15, 433.

15. Troll, W., and Cannan, R. K., *J. Biol. Chem.*, 1953, v200, 803.

Received April 3, 1956. P.S.E.B.M., 1956, v92.

Isolation of Chondroitinsulfuric Acid from the Cock's Comb.* (22396)

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Since both chondroitinsulfuric acid (CSA) and hyaluronic acid (HA) exist concomitantly in the skin of man and animals it seemed reasonable to suspect that the ground substance of the cock's comb also contains both substances. The isolation of HA has been reported by Boas(1) but the possible presence of sulfated mucopolysaccharides in the cock's comb appears to be unexplored. The present paper concerns the isolation and characterization of CSA from cock's combs.

Methods. Fresh combs† were freed of the outer corny and epithelial layers by means of a scalpel and ground in an electric grinder. A total of 293 g of ground comb was defatted with acetone and dried at room temperature, yielding 35 g of acetone-defatted dry material. The mucopolysaccharides were extracted by a method previously described for skin(2). The crude polysaccharide mixture which weighed 2.4 g gave values of 2.85 and 26.6%, respectively, for nitrogen and hexosamine. This mixture was subjected to zone electrophoresis on a slab of Celite filter-aid for the separation of HA and CSA fractions (2).

Results. From the slower migrating fraction, 1.5 g of HA was isolated. Chemical analyses demonstrated nitrogen, uronic acid, and hexosamine in a molecular ratio of 1.16, 1.00, and 0.93 respectively.

The faster moving fraction proved to be analytically identical with the CSA isolated from rabbit skin(2). A total of 105 mg was isolated. Nitrogen, hexosamine, uronic acid (as determined by the carbazole reaction of Dische(3)), and sulfur analyses indicated molecular ratios of 1.02, 1.00, 0.67, and 1.00, respectively. The CSA fraction of the cock's comb, like that of rabbit skin(2), is partially resistant to hydrolysis by testicular hyaluronidase as measured by a reduction in turbidity(4), since after incubation with the enzyme the optical density was decreased by only 26%

In neutral solutions, the $[\alpha]_D^{24} = -56°$ for the CSA fraction of the cock's comb. This value agrees with that of the corresponding fraction from rabbit(2) and from pig and calf skins(5).

The hexosamine of the CSA fraction was identified as galactosamine by the method of Stoffyn and Jeanloz(6).

Discussion. Since a considerable portion of the cock's comb is connective tissue it may prove of value as a tool for metabolic studies. A high percentage, 4.7%, of the dry weight was actually isolated as HA and CSA. The relative proportions of the isolated mucopolysaccharides are quite different from those found in rat skin(7). The preponderant mucopolysaccharide in the comb is HA, thus confirming the observation of Boas(1) regarding the presence of large quantities of HA in the cock's comb. While CSA represents only a small fraction of the total mucopolysaccharides, larger quantities were isolated from the cock's comb than were found in the rat skin, using an isotope dilution technic(7). In view of the partial hydrolysis of the CSA fraction by testicular hyaluronidase and the low carbazole values for uronic acid, the CSA fraction in rooster combs appears to be a mixture similar to that in rabbit skin(2).

Summary. 1. Hyaluronic acid (HA) and chondroitinsulfuric acid (CSA) were isolated from the combs of roosters in a ratio of 15 to 1. 2. The CSA fraction from combs appears to be similar to that isolated from rabbit skin.

* This investigation was aided by grants from Chicago Heart Assn. and Variety Club of Illinois.

† Combs from assorted breeds of roosters were supplied by Palo Products Co., Chicago.

We are indebted to Kathryn F. Dewey and Julio Ludowieg for technical assistance.

1. Boas, N. F., *J. Biol. Chem.*, 1949, v181, 573.

2. Schiller, S., Mathews, M. B., Jefferson, H., Ludowieg, J., and Dorfman, A., *ibid.*, 1954, v211, 717.
3. Dische, Z., *J. Biol. Chem.*, 1947, v167, 189.
4. Mathews, M. B., Roseman, S., and Dorfman, A., *J. Biol. Chem.*, 1951, v188, 327.
5. Meyer, K., and Rapport, M. M., *Science*, 1951, v113, 596.
6. Stoffyn, P. J., and Jeanloz, R. W., *Arch. Biochem. Biophys.*, 1954, v52, 373.
7. Schiller, S., and Dorfman, A., to be published.

Received April 3, 1956. P.S.E.B.M., 1956, v92.

Comparative Effects of Periodic Bleedings on Serum Glycoprotein Concentrations in Guinea Pig and Rat.* (22397)

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Increased concentrations of the carbohydrate-containing proteins of serum have been reported for a wide variety of physiologic and pathologic states(1). Precise information relative to the physiologic mechanisms involved in their formation or function is, however, still not available(2). Inasmuch as serial determinations are necessary in many experimental studies, the present investigation was undertaken to determine the effect of repeated bleedings, at weekly intervals on the serum concentrations of total glycoprotein, seromucoid and total protein, and on the hematocrit and hemoglobin values of blood in normal guinea pigs and rats.

Materials and methods. Guinea pigs. Adult, male, hybrid guinea pigs were housed 2 per cage and were maintained on a diet of Purina rabbit pellets, supplemented with lettuce, and tap water, *ad lib. Rats.* Adult, male, Sprague-Dawley rats were housed 2 per cage and were maintained on a diet of Purina laboratory chow and tap water, *ad lib. Bleeding.* The animals were bled by cardiac puncture, under light ether anesthesia, at weekly intervals for a total of 5 bleedings. At each bleeding 1.3 ml of blood per 100 g of body weight were removed, the sample representing approximately 20% of the total blood volume(3). Several animals died from the effects of the repeated hemorrhages. Data are presented for those that survived the experimental period. *Chemical analyses.* Total serum glycoprotein, seromucoid, total serum protein, hemoglobin, and hematocrit values were determined by methods previously reported(4,5). *Statistical analyses.* The mean, standard error of the mean, t, and probability values were determined by standard statistical procedures(6).

Results. Summaries of the results of the chemical analyses and hematologic data for the guinea pig and for the rat are presented in Tables I and II respectively.

The animals of both species continued to gain weight throughout the course of the experiment. A slight stimulation of growth followed the first bleeding; however, the average weekly weight increment gradually decreased.

No statistically significant increases occurred in the concentration of the serum components in comparison with base line values. In the guinea pig, a significant decrease was observed in the total serum glycoprotein at the third bleeding. Significant decreases in seromucoid levels occurred in the rat at the third and fourth bleedings. The diminished concentrations of seromucoid were reflected in significantly lower seromucoid-total glycoprotein ratios. Although a significant hypoproteinemia was exhibited by the rat at the second bleeding, total serum protein values

* Supported by research grants from National Cancer Institute, N. I. H. and the Los Angeles County Tuberculosis and Health Assn.

TABLE I. Effects of Periodic Bleedings on Serum Glycoprotein Concentrations in the Guinea Pig.

Bleeding No.	1	2	3	4	5
No. of animals	18	18	18	18	18
Wt (g)*	769 ± 30.0	792 ± 31.4	808 ± 30.5	821 ± 30.1	831 ± 23.6
Total serum glycoprotein (mg %)*	117 ± 2.2	114 ± 2.5	109 ± 2.1†	114 ± 2.0	115 ± 2.4
Seromucoid (mg %)*	34 ± .5	35 ± 1.2	33 ± 1.0	34 ± .8	34 ± .8
Total serum protein (g %)*	4.9 ± .07	5.0 ± .09	4.8 ± .09	5.0 ± .06	5.1 ± .08
Total glycoprotein ÷ total protein × 100 (%)*	2.4 ± .03	2.3 ± .03	2.3 ± .04	2.3 ± .03	2.3 ± .04
Seromucoid ÷ total glycoprotein × 100 (%)*	29.0 ± .48	30.7 ± .57	30.3 ± .78	29.8 ± .45	29.6 ± .37
Hematocrit (%)*	45 ± .7	40 ± .9‡	40 ± .7‡	40 ± .7‡	42 ± .5‡
Hemoglobin (g %)*	14.7 ± .25	13.6 ± .35†	13.3 ± .23‡	14.3 ± .29	13.4 ± .21‡

* Including stand. error of mean.

Statistically significant differences from baseline values are indicated:
† P = <.05 >.01. ‡ P = <.01.

were in the normal range at subsequent hemorrhages. Total glycoprotein-total protein ratios did not diverge from the normal in either species.

Statistically significant decreases occurred in hematocrit and hemoglobin levels in both species. In the guinea pig, the reductions were most pronounced following the initial bleeding. Repeated hemorrhages, however, had a much greater effect on the blood values of the rat. The diminution in both hemoglobin and hematocrit concentrations at the termination of the study was twice as great as that of the guinea pig.

Discussion. The restoration of the serum glycoproteins and proteins to essentially normal levels in the interval between bleedings, notwithstanding decreased hematocrit and hemoglobin values, is indicative of a rapid rate of synthesis. Although transient decreases occurred in the concentrations of different serum components, the response of both species to periodic bleedings was similar.

The results demonstrate that blood samples may be obtained from adult, male guinea pigs and rats at weekly intervals without highly significant alterations in the concentrations of the serum glycoproteins. It must be recognized, however, that the bleeding regimen engenders a mild anemia.

The present data are in accord with the conclusions of Robscheit-Robbins *et al.*(7) with regard to the relative rates of restoration of the serum proteins and hemoglobin. They observed in "doubly depleted" dogs that the serum proteins returned to normal levels much more rapidly than hemoglobin values.

The existence of pronounced species differences in serum glycoprotein concentrations is indicated by a comparison of the baseline values in Tables I and II. This observation has been previously reported(8) and has been confirmed in the current study.

Summary. The effects of repeated bleed-

TABLE II. Effects of Periodic Bleedings on Serum Glycoprotein Concentrations in the Rat.

Bleeding No.	1	2	3	4	5
No. of animals	20	20	20	20	20
Wt (g)*	338 ± 7.8	356 ± 9.2	365 ± 9.0	374 ± 10.1	376 ± 11.5
Total serum glycoprotein (mg %)*	155 ± 3.8	156 ± 3.2	163 ± 2.2	152 ± 2.4	160 ± 3.3
Seromucoid (mg %)*	22 ± 1.7	21 ± 1.2	17 ± .6†	17 ± .6†	21 ± 1.9
Total serum protein (g %)*	6.3 ± .08	6.0 ± .10†	6.4 ± .09	6.3 ± .12	6.2 ± .08
Total glycoprotein ÷ total protein × 100 (%)*	2.5 ± .05	2.6 ± .04	2.6 ± .03	2.4 ± .03	2.6 ± .05
Seromucoid ÷ total glycoprotein × 100 (%)*	14.2 ± .93	13.5 ± .63	10.4 ± .36‡	11.2 ± .33‡	13.1 ± .93
Hematocrit (%)*	47 ± .5	44 ± .5‡	44 ± .6‡	43 ± .4‡	41 ± .7‡
Hemoglobin (g %)*	15.1 ± .21	14.2 ± .17‡	14.3 ± .26†	13.6 ± .21‡	12.5 ± .31‡

* Including stand. error of mean.

Statistically significant differences from baseline values are indicated:
† P = <.05 >.01. ‡ P = <.01.

ings at weekly intervals on the serum concentrations of total glycoprotein, seromucoid, and total protein and on the hemoglobin and hematocrit values of blood have been investigated in adult, male guinea pigs and rats. No significant increases occurred in levels of the serum components. Transient decreases were observed in the total serum protein and seromucoid of the rat and the total serum glycoprotein of the guinea pig. Statistically significant decreases occurred in hematocrit and hemoglobin values following the first and ensuing hemorrhages.

1. Winzler, R. J., *Methods of Biochemical Analysis*, v2, Interscience Publishers, Inc., New York, 1955, 279.

2. Weimer, H. E., Redlich-Moshin, J., and Nelson, E. L., *J. Immunol.*, 1955, v74, 243.

3. Burke, J. D., *Physiol. Zool.*, 1954, v27, 1.

4. Weimer, H. E., and Moshin, J. R., *Am. Rev. Tuberc.*, 1953, v68, 594.

5. Weimer, H. E., Voge, M., Quinn, F. A., and Redlich-Moshin, J., PROC. SOC. EXP. BIOL. AND MED., 1955, v90, 494.

6. Snedecor, G. W., *Statistical Methods*, 4th edition, Iowa State College Press, Ames, 1946.

7. Robscheit-Robbins, F. S., Miller, L. L., and Whipple, G. H., *J. Exp. Med.*, 1945, v82, 311.

8. Weimer, H. E., Carpenter, C. M., Redlich Moshin, J., Little, M. S., and Nelson, E. L., *Physiol Zool.*, 1954, v27, 341.

Received April 3, 1956. P.S.E.B.M., 1956, v92.

Localization of Specific Cholinesterase About the Eccrine Sweat Glands of Human Volar Skin.* (22398)

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Eccrine sweating of the palms and soles in man occurs almost exclusively in response to non-thermal stimuli, usually emotional or mental(1); the response to heat is minimal and delayed(2). In sharp contrast to this, eccrine sweating of the rest of the skin is due primarily to thermal stimulation(1). Indeed, emotional and thermogenic sweating have been regarded as separate functions controlled by different mechanisms(3). The idea of different control mechanisms is partially supported by published pharmacologic evidence. On the one hand the glands of the general skin are stimulated to secrete sweat by cholinergic drugs and are inhibited by anticholinergic drugs but not by antiadrenergic drugs (4). These results accord with the demonstrated cholinergic innervation of the glands of the general skin(4). On the other hand the glands of the palms and soles have been reported to show little sweating in response to cholinergic drugs(5,6) and a reduction of spontaneous sweating in response to dibenamine, a potent adrenergic blocking agent(7). A possible inference, therefore, is that the volar glands have primarily an adrenergic innervation and the general skin glands have a cholinergic innervation. More recent studies, however, seem to indicate that the volar glands are also specifically stimulated by cholinergic drugs in the same concentrations as are effective for the general skin(8,9). Also, mental sweating of the palms was not inhibited by adrenergic blocking agents but was readily inhibited by the anticholinergic substance atropine(8). Furthermore, systemic administration of epinephrine does not evoke eccrine sweating(7), although local injection into the skin irregularly elicits the appearance of local sweat in both volar and general skin(4). This latter effect may be due to the stimulation of the myoepithelial cells surrounding the sweat gland tubules causing them to contract and eject preformed

* This investigation was supported by Research and Development Division, Office of Surgeon General, Department of Army, under Contract No. DA-49-007-MD 553, and Research Grant (C-2434) from National Cancer Institute, N. I. H., and Public Health Service.

sweat. The ensuing refractory state (1–24 hours) in the presence of generalized sweating is consistent with this interpretation(12). A similar state exists in the apocrine sweat gland where sweating consists of ejection by myoepithelial contraction in response to adrener

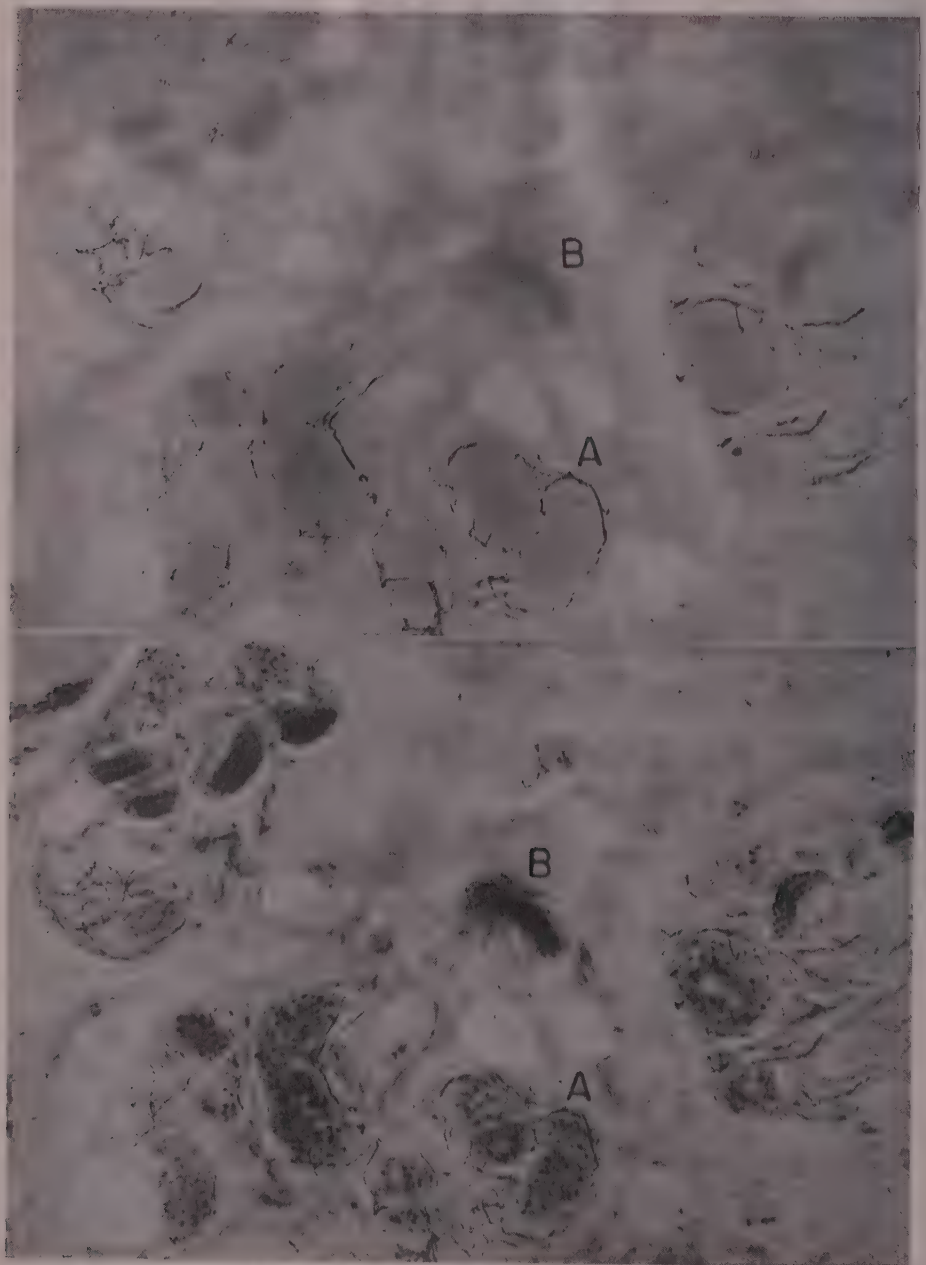

FIG. 1. Photomicrographs showing a specific cholinesterase in nerve fibers to secretory tubules of volar eccrine sweat glands (A), and absence of such fibers about eccrine ducts (B). Upper photograph—section counterstained lightly with eosin; lower—adjacent serial section counterstained with hematoxylin and eosin. Mag. × 100.

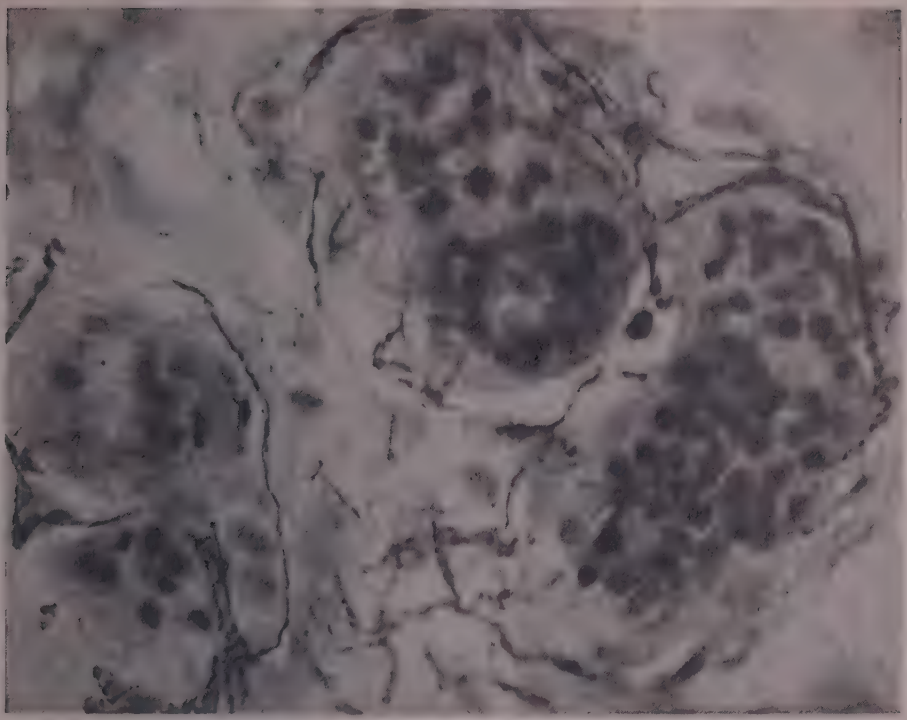

FIG. 2. View of eccrine sweat gland tubules under higher magnification. Observe nerve fibers containing specific cholinesterase. Section counterstained with hematoxylin and eosin. Mag. × 360.

gic stimulation; this is followed by a refractory period of 24-48 hours(13).

It is apparent that the possible existence of 2 different control mechanisms, an adrenergic for the volar glands and a cholinergic for the general skin glands, cannot be established on the basis of the conflicting pharmacologic evidence. It is first necessary to ascertain the nature of the innervation of the volar glands. There is no stain specific for adrenergic fibers. Cholinergic nerve fibers, however, can apparently be identified by means of Koelle's histochemical technic which discloses the localization of the enzyme, specific (true, aceto-, acetyl-) cholinesterase (ChE) in the membrane of the cholinergic fibers(10). This method was here employed to ascertain whether or not the volar sweat glands have a cholinergic innervation.

Materials and methods. Biopsies were obtained from the volar skin of the great toes of 6 healthy white male volunteers (22-26 years of age) after block anesthesia of the digits with 1% procaine. Immediately after excision the skin specimens were placed on dry ice. Frozen sections were cut at 10 μ thickness and placed on glass slides. They were then carried through the histochemical method of Koelle according to the latest modifications described for the localization of specific and non-specific cholinesterase activity(10). Acetylthiocholine and butyrylthiocholine were used as substrates and 10^{-10} M diisopropylfluorophosphate was employed to inhibit selectively the non-specific ChE of the appropriate sections.

Results. Specific cholinesterase was noted in many nerve fibers about the secretory tubules of the eccrine sweat glands (Fig. 1 and 2) and about the digital arteriovenous anastomoses(11). The enzyme was absent about the ductal portions of the eccrine sweat glands (Fig. 1B) and about all other cutaneous vessels. The control sections for non-enzy-

matic staining and other esterases were negative.

The cholinergic fibers appeared to terminate, if not on, then in close proximity to the secretory cells. It was not possible on the basis of these sections, however, to exclude the myoepithelial cells of the gland tubule as a possible site for the termination of the fibers.

Discussion. The above evidence demonstrates that the volar eccrine sweat glands have the same cholinergic innervation as the eccrine glands of the general skin. This information is in accord with the latest pharmacologic evidence that both volar and general skin glands respond similarly to cholinergic and other drugs(8,9). Hence it is necessary to account for the differential specificity of stimuli, non-thermal for the volar glands and thermal for the general skin glands, by a mechanism other than differential innervation. The differentiation as to effective stimulus must be traced back to the location in the central nervous system of the neurons which initiate the discharge of impulses which ultimately effect the secretion of sweat. These loci may be the cortex for impulses to the glands of the palms and soles, and the hypothalamus for impulses to the glands of the general skin surface.

Summary. By means of Koelle's histochemical technic for cholinesterase, the same cholinergic innervation was found for the eccrine sweat glands in the volar skin of the human toe as occurs for the glands in the general skin of the body. This evidence, coupled with recent pharmacologic results in the literature, was taken to indicate that non-thermal sweating in the palms and soles was due, not to a hypothetical adrenergic innervation of volar glands, but to a primary control center in the central nervous system (possibly the cortex) other than the control center (the hypothalamus) for thermal sweating.

We are grateful for kindness and suggestions of Dr. George W. Molnar, and to Miss Norma J. Simpson for technical assistance. Photographs were taken by Mr. Leo Goodman.

1. Kuno, Y. *The Physiology of Human Perspiration,* J. A. Churchill, London, 1934.

2. Ikeuchi, K., and Kuno, Y., *J. Orient. Med.,* 1927, v7, 67, 106.

3. Sulzberger, M. B., and Herrmann, F. *The Clinical Significance of Disturbances in the Delivery of Sweat,* Chas. C. Thomas Co., Springfield, Ill., 1954.

4. Rothman, S., *The Physiology and Biochemistry of the Skin.* Univ. of Chicago Press, Chicago, Ill., 1954.

5. Randall, W. C., *J. Clin. Invest.,* 1946, v25, 761.

6. Macht, M. R., and Bader, M. E., *J. Appl. Physiol.,* 1948, v1, 205.

7. Haimovici, H., *J. Appl. Physiol.,* 1950, v2, 512.

8. Chalmer, I. M., and Keele, C. A., *Brit. J. Dermat.,* 1952, v64, 43.

9. Sonnenschein, R. R., Kobrin, H., Janowitz, H. D., and Grossman, M. I., *J. Appl. Physiol.,* 1951, v3, 573.

10. Koelle, G. B., *Pharmacol. and Exp. Therap.,* 1951, v103, 153.

11. Hurley, H. J., Jr., and Mescon, H., *J. Applied Physiol.,* in press.

12. Sonnenschein, R. R., Kobrin, H., and Grossman, M. I., *Am. J. Physiol.,* 1949, v159, 591.

13. Shelley, W. B., and Hurley, H. J., Jr., *J. Invest. Dermat.,* 1953, v20, 285.

Received April 5, 1956. P.S.E.B.M., 1956, v92.

Effect of Pitressin on Adrenocortical Activity in Man. (22399)

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Vasopressin has been postulated to be the neurohormone responsible for enhanced ACTH release in acute stress situations (1-3). Sobel, *et al.*(4), have shown that the guinea pig excretes an increased quantity of neutral reducing lipids following parenteral administration of either posterior pituitary extract or commercial vasopressin (Pitressin). Intravenous administration of Pitressin in rats has been shown to result in ACTH discharge as evidenced by depletion of adrenal ascorbic acid(3). Using an *in vitro* method for the study of ACTH release by culture of the rat anterior pituitary gland, Guillemin and Hearn(5) have shown that incubation of the gland with Pitressin results in a significantly increased release of ACTH.

The present study was undertaken to determine the effect of an intravenous infusion of Pitressin on the release of ACTH as evidenced by increased 17-hydroxycorticosteroid production in human subjects.

Methods. Three male and 4 female normal subjects, ranging from 18 to 21 years of age, were studied. The test procedure consisted of determining free 17-hydroxycorticosteroid concentrations in plasma of blood samples drawn immediately before, and at intervals during and after, a 2-hour infusion of commercially prepared Pitressin† in normal saline. The amount of Pitressin infused ranged from 6.8 to 30.0 units. Blood samples were drawn at 60-minute intervals for 5 consecutive periods; then 2 samples were drawn at 120-minute intervals. Breakfast was served following the 2-hour infusion period. Free plasma and total urinary 17-hydroxycorticosteroid concentrations were determined by the method of Silber and Porter(6), as modified by Peterson, *et al.*(7). Blood for eosinophil counts was obtained at the beginning of infusion (8 A. M.) and 4 hours later (12 M.). Duplicate eosinophil counts were made in a Speirs-Levy eosinophil counting slide with Randolph's solution as diluting fluid. Control data were obtained with a 2-hour infusion of a similar volume of saline.

Results. Fig. 1 shows the mean changes in plasma 17-hydroxycorticosteroid concentration over the 8-hour period in which they were determined. The plasma 17-hydroxycorticosteroid levels associated with the infusion of Pitressin are significantly‡ higher than the saline control values during the first 4 hours of the test.

The possibility that ACTH contamination of the Pitressin preparation was responsible for the rise in the plasma 17-hydroxycorticosteroids was considered and two procedures were employed to ascertain whether this was the case. The first procedure consisted of inactivating the Pitressin by the method of Smith, *et al.*(8), and administering it to 2 subjects to determine whether a rise in plasma 17-hydroxycorticosteroids resulted. The second procedure consisted of calculating the amount of ACTH administered to these subjects using the figure of 0.1 milliunit of ACTH per unit of Pitressin(9). Neither of these procedures caused a significant rise in the plasma levels of 17-hydroxycorticosteroids.

The 4-hour eosinophil counts were significantly lower when Pitressin was administered than when saline alone was infused. The 12-hour urinary excretion of 17-hydroxycorticosteroids on the Pitressin test day averaged 34% higher than on the saline test day. This difference, however, is not significant ($P < 0.1$).

Blood pressure and pulse data are shown graphically in Fig. 2. No significant changes in blood pressure were associated with administration of Pitressin. Pulse rate was de-

* NIH, Public Health Service, U. S. Dept. of Health, Education, and Welfare.

† Parke, Davis & Co., lot nos. R66F and L909E.

‡ The term "significant" is used to indicate statistical significance at 1% level of confidence as determined by the t-test of Fisher.

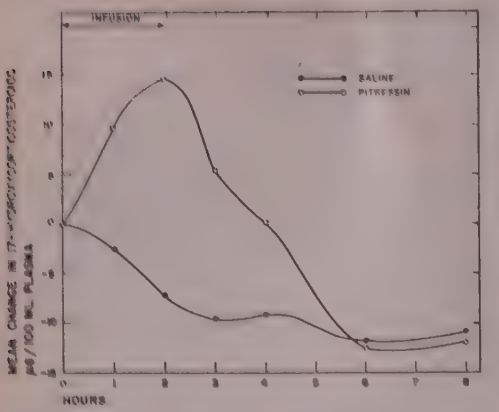

FIG. 1. Effect of Pitressin on plasma 17-hydroxycorticosteroid concentration. Individual points represent mean values for group of 7 young, normal subjects receiving between 6.8 and 30 units of Pitressin in a 2-hr intravenous infusion.

creased significantly during the second hour of Pitressin infusion.

Discomfort in the form of anorexia, moderate nausea, abdominal cramping sensations, and defecatory urges were noted frequently. Three of the 4 female subjects noted uterine cramping. Vomiting did not occur in any patient. No attempt was made to quantify the discomfort. Marked facial pallor, coldness of the hands, and diminution of the radial artery pulsations, frequently to the point of imperceptibility, were noted. In spite of this remarkable diminution in peripheral pulsation, no significant changes in the auscultatory blood pressures were noted.

Comments. The fact that intravenous administration of Pitressin is associated with a marked rise in plasma 17-hydroxycorticosteroid concentration is consistent with the suggestion that vasopressin is the neurohormone involved in ACTH stimulation in acute stress situations. However, the mechanism by which this stimulation is effected is not apparent. As was noted, administration of Pitressin caused the subjects considerable discomfort which could result in a release of ACTH. In this regard, it is of interest to note that addition of Pitressin to the nutrient fluid of rat anterior pituitary glands maintained in organ culture caused a release of ACTH, indicating that such a release is possible by direct action of Pitressin on the anterior pituitary gland(5).

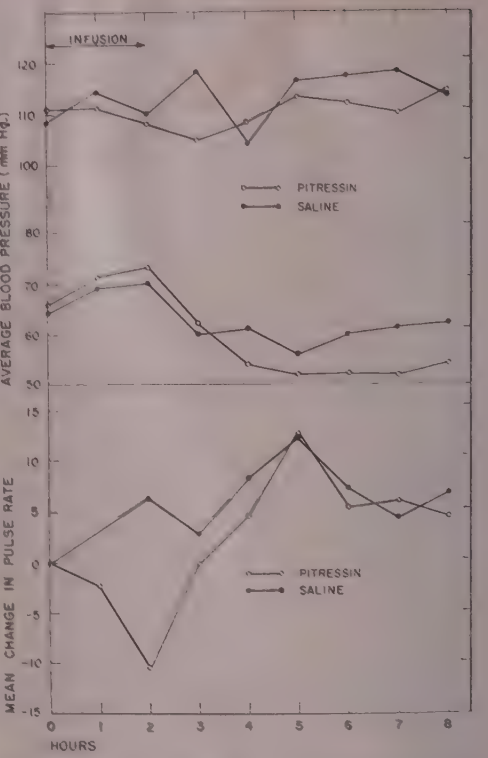

FIG. 2. Effect of Pitressin on pulse rate and blood pressure. Individual points represent mean values for group of 7 young, normal subjects receiving between 6.8 and 30 units of Pitressin in a 2-hr intravenous infusion.

Summary. Intravenous administration of Pitressin causes a significant increase in plasma 17-hydroxycorticosteroid concentration in human subjects.

The authors wish to express appreciation to Daniel W. Clink and Francis A. Esposito for technical asistance.

1. Rothballer, A. B., *Anat. Rec.*, 1953, v115, 21.

2. Mirsky, I. A., Stein, M., and Paulisch, G., *Endocrinology*, 1954, v55, 28.

3. McCann, S. M., and Brobeck, J. R., PROC. SOC. EXP. BIOL. AND MED., 1954, v87, 318.

4. Sobel, H., Levy, R. S., Marmorston, J., Schapiro, S., and Rosenfeld, S., *ibid.*, 1955, v89, 10.

5. Guillemin, R., and Hearn, W. R., *ibid.*, 1955, v89, 365.

6. Silber, R. H., and Porter, C. C., *J. Biol. Chem.*, 1954, v210, 923.

7. Peterson, R. C., Wyngaarden, J. B., Guerra, S. L., Brodie, B. B., and Bunim, J. J., *J. Clin. Inv.*, 1955, v34, 1779.

8. Smith, E. L., Sayers, George, Ghosh, B. N., and Woodbury, D. M., PROC. SOC. EXP. BIOL. AND MED., 1952, v79, 27.

9. Sayers, G., personal communication.

Received April 5, 1956. P.S.E.B.M., 1956, v92.

Effect of Oxytocin on Adrenocortical Activity in Man. (22400)

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In evaluation of the effect of hypothalamic neurohormones on ACTH release in man, it has been shown that intravenous administration of commercial vasopressin (Pitressin) causes a significant rise in plasma 17-hydroxycorticosteroid concentration(1). In view of the fact that both oxytocin and vasopressin are present in the hypothalamus as well as in the posterior pituitary gland(2-5), we have extended our initial observation to evaluate the effect of the intravenous administration of oxytocin on the plasma 17-hydroxycorticosteroid concentrations. In the present study, both commercial oxytocin (Pitocin)† and a highly purified preparation of oxytocin‡ have been employed.

Methods. Four male and 4 female, normal subjects, ranging from 18 to 21 years of age, were studied. The general procedure and laboratory analyses have been previously described(1). Saline was the control infusion for the Pitocin study and saline plus oxytocin diluent was the control infusion for the oxytocin study. All infusions were given from 8 A. M. to 10 A. M.

Results. Effect of Pitocin. Fig. 1 shows the mean change in the plasma 17-hydroxycorticosteroid concentration associated with the infusions of Pitocin and the control solution. There is no significant difference between the 2 curves. Pitocin was without effect on the 12-hour urinary excretion of 17-hydroxycorticosteroids and on the number of circulating eosinophils. Pulse and blood pressure recorded hourly for the 8-hour period showed no difference between the days of Pitocin and saline infusions.

Effect of Highly Purified Oxytocin. Since Pitocin has only 50-70% of the purity of du Vigneaud's highly purified oxytocin(6), it was deemed necessary to test the latter preparation. Four individuals were infused with 9 units of highly purified oxytocin in saline on one day and with a comparable volume of the control solution on another day. No significant difference between plasma 17-hydroxycorticosteroid levels during the 2 tests was noted (Fig. 2).

In another series of observations, 6 subjects were infused with an increased amount

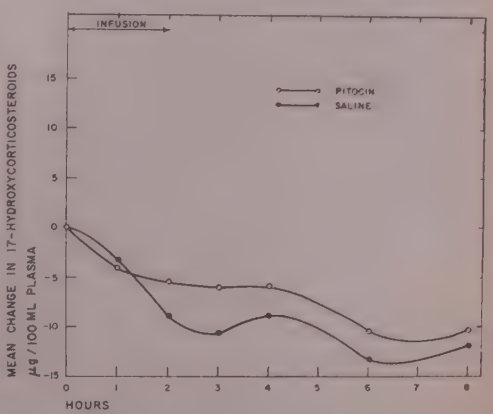

FIG. 1. Effect of Pitocin on plasma 17-hydroxycorticosteroid concentration. Individual points represent mean values for group of 7 young, normal subjects receiving between 22.6 and 34.6 units of Pitocin in a 2-hr intravenous infusion.

* NIH, Public Health Service, U. S. Dept. of Health, Education, and Welfare.

† Parke, Davis & Co., lot nos. S-1970 and M-307M.

‡ Dr. Vincent du Vigneaud, Cornell University Medical College, kindly supplied us with this preparation of oxytocin.

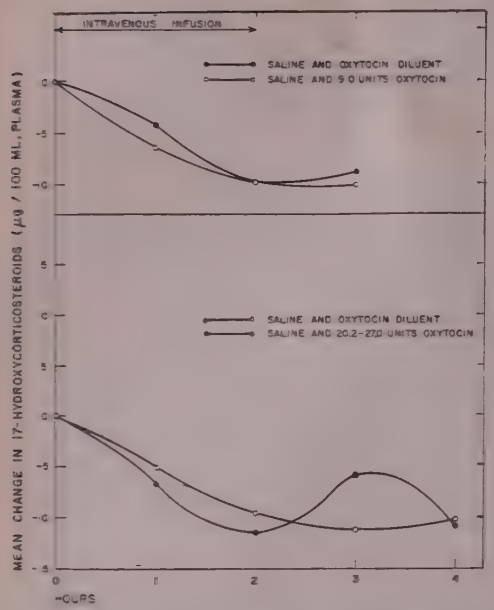

FIG. 2. Effect of infusion of highly purified oxytocin in saline on plasma 17-hydroxycorticosteroid concentration. Upper graph represents mean values of 4 young, normal subjects given 9 units of highly purified oxytocin in saline infusion and saline infusion containing oxytocin diluent. All infusions were administered at 1 ml/min. for 2 hr. Lower graph represents mean values of 6 young, normal subjects given 21.7 to 27 units of highly purified oxytocin in saline infusion and saline infusion containing oxytocin diluent. All infusions administered at approximately 3 ml/min. for 2 hr.

of oxytocin (20.2 - 27 units). Again, the control data were obtained using a comparable volume of saline plus oxytocin diluent. Infusion of the larger amount of oxytocin had no significant effect on the plasma 17-hydroxycorticosteroid levels (Fig. 2). The secondary rise in plasma 17-hydroxycorticosteroids that occurred during the hour following the termination of the oxytocin infusion is not significant ($P<0.2$). The 12-hour urinary excretion of 17-hydroxycorticosteroids and the fall in eosinophils were not significantly different during this oxytocin test and the control test.

Comments. It is apparent that neither Pitocin nor highly purified oxytocin has any stimulatory effect on ACTH production in this experimental situation. This is of interest in view of the structural similarity of oxytocin and vasopressin and the fact that there is some degree of overlap in their physiologic effects(4).

In initial observations on the effect of intravenous administration of oxytocin on ACTH release, it appeared that there was a secondary rise in plasma 17-hydroxycorticosteroid concentration an hour after the infusion had been terminated. However, this secondary rise was shown convincingly by only 3 of the 6 individuals and, therefore, was not statistically significant.

Conclusions. Intravenous infusion of either Pitocin or highly purified oxytocin is not associated with increased plasma concentrations or urinary excretion of 17-hydroxycorticosteroids.

The authors wish to express appreciation to Daniel W. Clink and Francis A. Esposito for technical assistance.

1. McDonald, R. K., and Weise, V. K., PROC. SOC. EXP. BIOL. AND MED., 1956, v92, 107.

2. Abel, J. J., *Bull. Johns Hopkins Hosp.*, 1924, v35, 305.

3. Hild, W., and Zetler, G., *Klin. Wochschr.*, 1952, v30, 433.

4. Bargmann, W., and Scharer, E., *Am. Scientist*, 1951, v39, 255.

5. van Dyke, H. B., Adamsons, K., Jr., and Engels, S. L., *Recent Progress in Hormone Research*, 1955, vXI.

6. McGinty, D. A., personal communication.

Received April 5, 1956. P.S.E.B.M., 1956, v92.

Elution of an Esterase from Antigen-Antibody Aggregates Treated with Human Complement.* (22401)

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It was reported recently(1) that partially purified first component of human complement (C′1) contains a pro-esterase. Adjustment of such a solution of purified C′1 to physiologic conditions of pH and ionic strength resulted in disappearance of the hemolytic activity of C′1 and appearance of anti-complementary and esterase activities. The complement-inactivating property was directed primarily toward the fourth component (C′4) and, to a lesser degree, toward the second component (C′2); esterase activity was observed with the synthetic substrates, p-toluenesulfonyl-L-arginine methyl ester (TAMe) and acetyl-L-tyrosine ethyl ester (AcTyEe) (2). A correlation existed between the time of disappearance of hemolytically active C′1 and the parallel appearance of both anti-complementary and esterase activities. In accordance with these observations and previous investigations(3-7), it was postulated that C′1 may be a pro-esterase.

It is the purpose of this paper to present further evidence for the pro-enzymatic role of C′1. Such evidence has been obtained by eluting from antigen-antibody aggregates, previously treated with fresh human serum, a fraction, representing less than 0.04% of the original serum nitrogen, which contains both esterase and anti-complementary activities. These properties are qualitatively similar to those obtained after "conversion" of purified C′1(1), and cannot be ascribed to any other known serum factor.

Materials and methods. Pre-formed *antigen-antibody aggregates*(6) were prepared by adding a solution of purified pneumococcal specific soluble substance, S-III† to purified antipneumococcal S-III rabbit serum† in the ratio of 1 mg S-III to 25 mg antibody N. The resulting suspension was brought to a final volume of 250 ml per mg S-III with 0.15 M NaCl. The mixture was stirred occasionally at 1° for 12-24 hrs. and then centrifuged at 4000 r.p.m. for 30 min. at 1°. The antigen-antibody precipitate was washed twice at 1° with 0.15 M NaCl of the same volume as the original suspension, stored at 1°, and used within 24 hrs. *Cholinesterase* activity of eluate preparations was determined by the method of Michel(8). *Hydrolysis of AcTyEe* and of acetyl-L-tryptophane ethyl ester (AcTrEe) was measured by the same technic used to measure *hydrolysis of TAMe*(1,9). *All other relevant materials and methods were presented previously*(1).

Results. 1. Preparation of Eluates. It was shown previously that antigen-antibody aggregates do not inactivate C′2 and C′4 in the absence of C′1(5,10). However, if antigen-antibody aggregates are first treated with serum reagents containing C′1 and washed free of occluded serum, they are then capable of inactivating C′2 and C′4 in the absence of additional C′1(6). This procedure of "activating" antigen-antibody aggregates by treatment with serum reagents containing C′1, designated the "Activation Stage"(6), is the basis for the elution experiments to be described. Fresh human serum was used as a source of C′1, and serum heated at 56° for 30 min. as a control reagent deficient in C′1.‡ Pre-formed antigen-antibody aggregates were treated with these serum reagents under con-

* Presented in part at 128th meeting of Am. Chem. Soc., Minneapolis, Minn., Sept. 14, 1955. This investigation was supported by grants from Lederle Laboratories Division, American Cyanamid Co., National Heart Institute of N. I H., Public Health Service, and The Cleveland Area Heart Soc.

† Kindly provided by Lederle Laboratories Division, American Cyanamid Co.

‡ Serum heated at 56° for 30 min. is also deficient in C′2 and, to a lesser degree, C′3. However, these components are not involved in the "Activation Stage"(6).

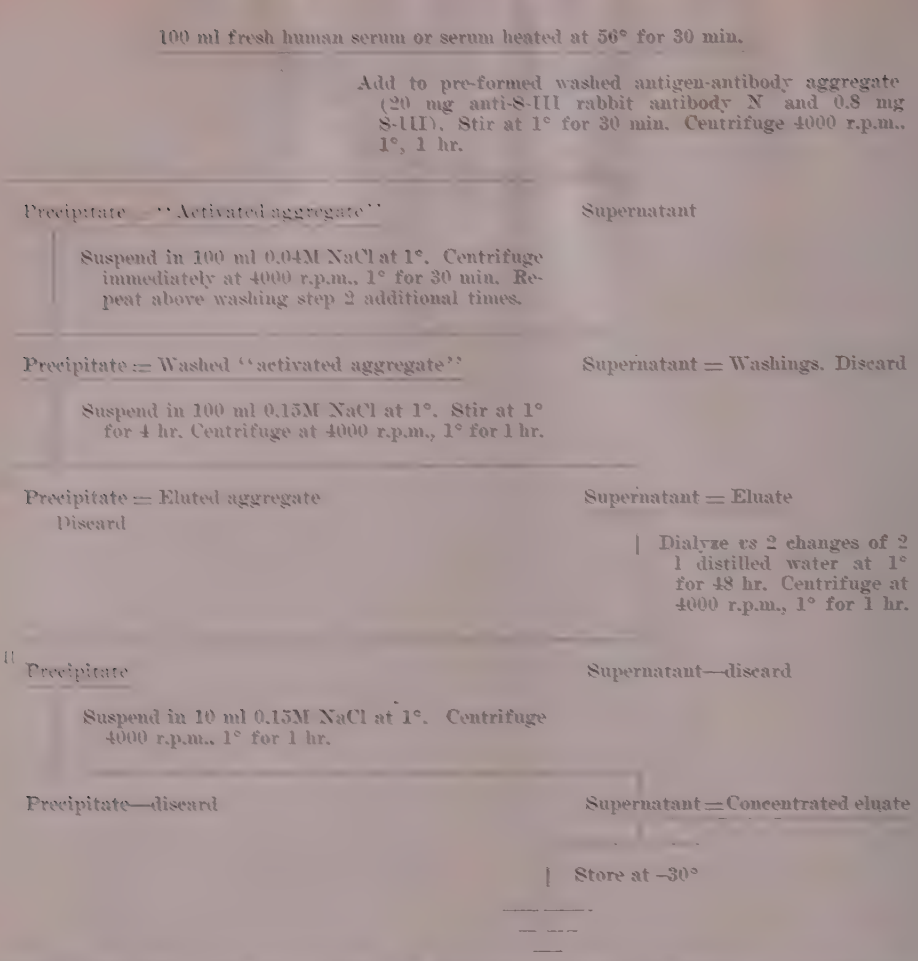

FIG. 1. Flow diagram for preparation of eluates from antigen-antibody aggregates treated with human serum reagents.

ditions identical to those described previously for the "Activation Stage"(6), washed with 0.04 M NaCl, and then eluted at 1° with 0.15 M NaCl. The eluates were dialyzed against distilled water, the small precipitates collected by centrifugation and dissolved in 0.15 M NaCl. A flow diagram of the entire procedure is shown in Fig. 1. The concentrated eluates did not contain measurable components of complement, properdin(11), acid or alkaline phosphatase,§ cholinesterase, prothrombin, proconvertin, proaccelerin, or thrombin. Spontaneous plasmin activity also could not be detected employing either fibrinogen or casein as substrates. Small amounts of Hageman factor(12) and of streptokinase-activable plasminogen were present. The latter could be demonstrated in the fibrinolytic assay; however, the amount was too small to be measured in the caseinolytic assay. Eluates prepared from fresh human serum and concentrated 10-fold over serum contained about 0.04 mg nitrogen/ml; with eluates prepared from serum heated at 56° for 30 min., the corresponding value was about 0.01 mg nitrogen/ml.

2. *Esterase and Complement-Inactivating Properties of Eluates.* The concentrated eluate derived from antigen-antibody aggregates

§ Kindly performed by Dr. J. W. Price.

TABLE I. Effect of Eluates AE and UE on Components of Complement in 0.75 ml R1.*

Eluate, ml	0.15M NaCl, ml	Inactivation of complement component† C'2	C'3	C'4
.01 AE	.24	0	0	60
.03 "	.22	35	0	80
.05 "	.20	35	0	95
.10 "	.15	50	0	100
.15 "	.10	70	0	100
.25 "	0	90	0	100
.25 UE	0	0	0	0

* R1 at pH 7.4, ionic strength 0.15, containing 2.5 × 10^{-3}M Ca^{++}, diluted 1/1.5 with respect to serum.

† Measured after incubation for 30 min. at 37°, based on complement component titers of R1 treated only with 0.25 ml of 0.15M NaCl.

treated with fresh human serum (designated AE) was highly anti-complementary because the preparation inactivated C'4 and, to a smaller degree, C'2. C'1 and C'3 were not significantly affected. The inactivation of C'2 and C'4 could be demonstrated by incubating AE with fresh serum or with R1 (the serum reagent deficient in C'1 but containing C'2, C'3 and C'4). Since plasmin does not inactivate C'2 and C'4 in R1(5), the use of this reagent minimized the possibility that the observed effects were due to activation of plasminogen to plasmin. In contrast to AE, the concentrated eluate derived from antigen-antibody aggregates treated with heated human serum (designated UE) did not inactivate complement or its components in serum or R1. A typical experiment demonstrating the effect of eluates AE and UE on components of complement in R1 at 37° is shown in Table I.

Eluate AE not only inactivated C'4 and C'2 but also had esterase activity against TAMe (Table II) and AcTyEe. No activity could be demonstrated against L-lysine ethyl ester (LEe) or AcTrEe. Eluate UE was inactive as an esterase.

Discussion. It has been shown that an eluate derived from antigen-antibody aggregates treated with fresh human serum (eluate AE) was capable both of inactivating C'4 and, to a lesser degree, C'2, and of hydrolyzing the synthetic esters, TAMe and AcTyEe. However, when antigen-antibody aggregates were treated with human serum which had been previously heated at 56° for 30 min., the corresponding eluate (UE) contained none of these activities. Thus, the factor which was adsorbed by aggregates is heat-labile. Two independent lines of evidence suggest the conclusion that this heat-labile factor is C'1.

First, previous investigations(5,6) showed that antigen-antibody aggregates could inactivate C'2 and C'4 in R1 only when the aggregates were treated with serum reagents rich in C'1. In the experiments described here, aggregates were treated with fresh serum (rich in C'1) or with heated serum (deficient in C'1) under conditions identical to those employed in a previous study(6). Since eluate AE inactivated C'2 and C'4 and eluate UE did not, it is concluded that C'1 must have been a factor adsorbed from unheated serum. Since C'1 activity is not demonstrable in the eluate, one inference is that C'1 is altered in some manner during adsorption by and elution from antigen-antibody aggregates.

Second, recent studies with partially purified human C'1(1) showed that a purified preparation of C'1 maintained its hemolytic activity as long as it was kept at pH 5.5, ionic strength 0.30. However, when the solution was brought to pH 7.4, ionic strength 0.15, it lost its C'1 activity and simultaneously acquired both anti-complementary and esterase activities. This preparation was designated "converted C'1" and its activities were strikingly similar to those of the active eluate (AE) described above. Similarly, purified C'1 maintained at pH 5.5, ionic

TABLE II. Esterase Activity of Eluates AE and UE. 1 ml TAMe,* 2.5 ml buffer.

Eluate, ml	0.15M NaCl, ml	Esterase activity,† .05N NaOH, ml
—	1.5	.027
.5 AE	1.0	.046
1.0 "	.5	.108
1.5 "	0	.152
1.5 UE	0	.011

* 0.4M TAMe dissolved in pH 7.4 phosphate buffer, ionic strength 0.15.

† Net micro-titration after 60 min. at 37°.

strength 0.30 appeared to be identical to the factor in fresh serum which was adsorbed by antigen-antibody aggregates.

Since "converted C'1" would be formed spontaneously under the conditions of adsorption and elution, a direct experiment employing antigen-antibody aggregates and purified C'1 is not as yet feasible. However, "converted C'1" and the active eluate have the following properties in common: esterase activity against TAMe and AcTyEe (2), but not against LEe or AcTrEe; ability to inactivate C'4 and, to a lesser extent, C'2; anti-complementary activity demonstrable both at 1° and 37°; esterase activity not demonstrable at 1°; absence of components of complement; activity independent of conversion of plasminogen to plasmin or of the presence or absence of properdin(11), prothrombin, proconvertin, proaccelerin, thrombin, or Hageman factor(12); absence of spontaneous plasmin activity, of cholinesterase, and of acid or alkaline phosphatase.

A detailed kinetic study of the esterase and complement-inactivating properties of "converted C'1" and of the active eluate is in progress. However, on the basis of evidence now available, the conclusion is warranted that C'1 probably exists in serum as a proenzyme which is activated by antigen-antibody aggregates. The immediate significance of this conclusion is two-fold: a long-standing postulate(13,14) that antigen-antibody aggregates activate one or more proenzymes in serum is substantiated, and the nature of one enzyme system is indicated; the mechanism of complement "fixation" by antigen-antibody aggregates is clarified. The significance of these observations in immunity and hypersensitivity remains to be determined.

Summary. An eluate (AE) prepared from antigen-antibody aggregates previously treated with fresh human serum contains esterase and anti-complementary activities. A corresponding eluate (UE) prepared from aggregates treated with serum heated at 56° for 30 min. does not contain these activities. The anti-complementary activity of AE is directed primarily against C'4; the esterase activity is demonstrable using TAMe and AcTyEe as substrates. Antigen-antibody aggregates adsorb C'1 or a factor closely resembling it, which upon elution appears to be identical to a previously described esterase derived from partially purified C'1. These results indicate that C'1 exists in serum as a proenzyme which is converted to an active esterase by antigen-antibody aggregates.

Thanks are due to Miss Joan E. Colopy for technical assistance.

1. Lepow, I. H., Ratnoff, O. D., Rosen, F., and Pillemer, L., PROC. SOC. EXP. BIOL. AND MED., 1956, v92, 32.

2. Unpublished experiments.

3. Pillemer, L., Ratnoff, O. D., Blum, L., and Lepow, I. H., *J. Exp. Med.*, 1953, v97, 573.

4. Lepow, I. H., Pillemer, L., and Ratnoff, O. D., *ibid.*, 1953, v98, 277.

5. Lepow, I. H., Wurz, L., Ratnoff, O. D., and Pillemer, L., *J. Immunol.*, 1954, v73, 146.

6. Lepow, I. H., and Pillemer, L., *ibid.*, 1955, v75, 63.

7. Lepow, I. H., abstracts 128th meeting Am. Chem. Soc., 1955, p43c.

8. Michel, H. O., *J. Lab. Clin. Med.*, 1949, v34, 1564.

9. Troll, W., Sherry, S., and Wachman, J., *J. Biol. Chem.*, 1954, v208, 85.

10. Pillemer, L., Seifter, S., and Ecker, E. E., *J. Exp. Med.*, 1942, v75, 421.

11. Pillemer, L., Blum, L., Lepow, I. H., Ross, O. A., Todd, E. W., and Wardlaw, A. C., *Science*, 1954, v120, 279.

12. Ratnoff, O. D., and Colopy, J. E., *J. Clin. Invest.*, 1955, v34, 602.

13. Bronfenbrenner, J., PROC. SOC. EXP. BIOL. AND MED., 1914, v12, 3.

14. Jobling, J. W., Petersen, W., and Eggstein, A. A., *J. Exp. Med.*, 1915, v22, 401.

Received April 9, 1956. P.S.E.B.M., 1956, v92.

Vitamin B_6 and Erythropoiesis in the Rat.* (22402)

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Vit. B_6 deficiency has been shown to result in anemia in several species including dogs, swine, and chicks(1). The anemia observed has been microcytic and hypochromic. Quite recently it has been reported that vit. B_6-deficient rats exhibit a decreased mean corpuscular hemoglobin and a slightly increased red cell count(2). In the latter experiments peripheral lymphocytes were found to be reduced in the vit. B_6-deficient rats. The data to be given in the present report indicate that vit. B_6 is definitely required for normal hemoglobin production by the rat.

Methods. Weanling Sprague-Dawley rats of both sexes were fed a basal vit. B_6-deficient diet consisting of vitamin-free casein, 18.6 g; sucrose, 67.4 g; lard, 8.0 g; cod liver oil, 2.0 g; salt mix (*J. Nutrition*, v14, 273), 4.0 g; choline chloride, 0.1 g; inositol, 10 mg; riboflavin, 0.8 mg; thiamine chloride, 0.5 mg; Ca pantothenate, 2 mg; nicotinic acid, 2 mg; 2-methyl-1, 4-napthoquinone, 0.44 mg. The animals were each given 3 mg of α-tocopherol acetate 2 times weekly. Control rats received this same diet supplemented with 10 mg of pyridoxine per kilo of diet. The animals were fed these diets for 15 weeks at which time complete blood counts were made on tail blood. There were 6 rats per group and the data were analyzed statistically by the "t" test.

Results. The data in Table I show that vit. B_6 deficiency resulted in an increase in red cell count and a decrease in hemoglobin, mean cell volume, and mean cell hemoglobin. The most striking change due to vit. B_6 deficiency was the reduction in mean cell hemoglobin. These data indicate that vit. B_6 is necessary for hemoglobin synthesis by the rat.

As shown by the data in Table II, vit. B_6 deficiency resulted in a significant lymphopenia and a striking increase in peripheral neutrophils.

TABLE II. Influence of Vit. B_6 Deficiency on Peripheral Leucocytes of Rats.

Animals	Total leucocytes	Lymphocytes	Neutrophils
	Thousands/μl		
Control	12.5	11.0	1.3
B_6-deficient	23.5	6.5	16.6
P*	<.01	<.01	<.01

* See footnote to Table I.

TABLE I. Influence of Vit. B_6 Deficiency on Peripheral Erythrocytes of Rats.

Animals	Erythrocytes, millions/μl	Hematocrit, ml/100 ml	Hemoglobin, g/100 ml	Mean cell vol, μ^3	Mean cell hemoglobin, $\mu\mu$g
Control	6.95	51	14.5	73	20.9
B_6-deficient	8.75	48	11.3	55	12.9
P*	<.01	<.1, >.05	<.01	<.01	<.01

* Probability that differences between control and B_6-deficient means are due to chance.

These data taken in conjunction with other reports in the literature demonstrate that the rat must be added to the list of animals requiring vitamin B_6 for normal erythropoiesis.

Summary. 1) Severe vit. B_6 deficiency in the rat resulted in an increased red cell count and in a reduction in hemoglobin, mean cell volume, and mean cell hemoglobin. The most striking effect was the reduction in mean cell hemoglobin in the deficient rats. The B_6-deficient rats exhibited lymphopenia and granulocytosis. 2) The data show that the rat must be added to the list of animals requiring vit. B_6 for normal hematopoiesis.

1. Cartwright, G. E., *Blood*, 1947, v2, 111.

2. Batchen, J. M., Cheesman, E. M., Copping, A. M., and Trusler, A. D., *Brit. J. Nutr.*, 1955, v9, 49.

* This work was supported by research grant No. A-721 from N.I.H., Public Health Service.

Received April 9, 1956. P.S.E.B.M., 1956, v92.

Topical Localization in Mouse of Radioactive Silver Oxide $[(Ag^{111})_2O]$ Introduced by Various Routes.* (22403)

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Previous work by West(1,2) has revealed that the short half-life radiosilver isotope Ag^{111}, injected in the form of a soluble salt (nitrate) intramuscularly or intravenously is taken up by blood leukocytes and carried into inflammatory body areas. It was presumed that combination of silver nitrate with tissue proteins resulting from the corrosive effect of this salt on tissues was a prerequisite for phagocytosis of radioactive material and its transportation in the blood stream by phagocytes.

It occurred to us that microscopic particles of an insoluble inorganic silver compound would be equally a target for phagocytes with the advantage of greater uniformity and of lack of corrosive action on tissues. Moreover, it was conceivable that this material introduced in the vicinity of tumor tissue will be carried by normal tissue lymphatics into lymph spaces of the tumor and stored by its phagocytic elements. Accordingly, we have injected radioactive silver oxide $(Ag^{111})_2O$ into subcutaneous tissue on the periphery of mouse tumors or into body serous cavities (peritoneal and pleural) containing solid tumor implants and exudate. Controls were normal mice injected with the same material in corresponding body areas by the same routes. The fate of injected material was investigated by taking autoradiographs from treated tumor bearing mice and controls and, moreover, by testing radioactivity (Geiger counts) of tumor tissue and of various organs from the mice of each series. The results illustrated the modification of radiosilver distribution in the body by the presence of tumor growth near the site of radiosilver injection.

* This work was supported by grant No. C2311 from the National Cancer Institute, NIH, and by a grant-in-aid from the American Cancer Society.

Material and methods. Radioactive Ag^{111} was separated in the form of nitrate from the radioactive palladium target (obtained from the pile at Oak Ridge A.E.C. Laboratories) by a procedure developed by William F. Clark; it was transformed into the oxide by treatment with sodium hydroxide. The material was washed several times with distilled water, suspended in the same at the ratio of 1.0 or 2.0 millicurie per 10 cc and vigorously shaken before each injection in order to break clumps into uniform particles. Doses of 0.1 or 0.2 mc were injected subcutaneously into mice carrying 7- to 15-day-old tumor growths on the back, the scalp or the hind leg; the dose was introduced in 5 fractions at various sites of subcutaneous tissue on tumor periphery and/or tumor bed. Similar doses were injected into pleural or peritoneal cavity bearing malignant tissue implants and exudate. The following tumor strains and mice strains were used: Sarcoma 180 and Sarcoma McGhee (isolated in our laboratories) in Swiss Albino strain, Carcinoma C_3H/BA in C_3H mice and Carcinoma EO771 in C57-6 mice. In controls (normal mice) similar doses were introduced into the same areas of the skin or the cavities. Twenty-four hours later, the animals were sacrificed: 2 or 3 mice of each series (10 to 15 mice) were used for autoradiography, while in the remaining mice the organs and tumor tissue were placed into tin cups, ground, suspended in a detergent solution, dried and tested by a scaling circuit in conjunction with a thin mica end-window Geiger tube. Subcutaneous tumors (mostly single spheric lumps) were cut each in 4 quarters, partly because they were bulky and contained large amounts of radioactivity and partly because this procedure afforded some information on distribution of radioactive material in the tumor tissue. Peritoneal and

TABLE I. Distribution of Radioactive $Ag^{111}O$ Injected by Various Routes into Normal and Tumor Bearing Mice.

Route of inj.	Geiger counts (avg of each series)										
	Liver	Spleen	Lung	Sto-mach	Small intes-tine	Colon	Kid-ney	Subcut. tissue at site of inj.		Tumor tissue (and intracavitary exudate)	
1. Subcut. in normal mice	1,465	140	31	53	45	46	39	4,569	1		
2. Subcut. into tumor periphery	461	67	99	97	116	109	102	604	3,028	2,517	3 7,060	4 6,974
3. Into normal pleural cavity	388	62	3,291	65	74	78	85	224			
4. Into pleural cavity with malignant growth	1,394	106	8,340	58	44	48	75	188		15,068	
5. Intraper. in normal mice	2,786	664	81	218	315	464	228	848			
6. Into peritoneal cavity bearing malignant growth	706	223	76	135	182	72	94	528		18,602	

Note: Counts in 3 groups each of 10 mice were pooled in each series. For technical reasons results are fully comparable within each series, but not between different series of mice (inj. by various routes).

pleural exudates were tested separately from intracavitary implants, but since this separation was not complete (exudate contained often fragments of implants) the results were pooled (as shown in Table I). For obtaining autoradiographs, each mouse was secured to a cardboard in a position that presented the best profile of the area of malignancy. It was then quick frozen to minimize the possibility of further metabolic activity. After freezing, the surface activity was measured by means of a precision counting rate meter in order to determine the required exposure time. The animals were then placed in pliofilm envelopes and secured to cardboard x-ray film. They were placed in a deep freeze for the required exposure time. At the end of this period each film holder and its contents were x-rayed intact; thus, the skeletal structure is included on the same film with the exposure due to isotope radiation. In other words, a photograph was taken showing localization of radioactive material on the background of an x-ray picture of the skeleton in the body of each animal.

Results. Autoradiographs 1-6 illustrate the tendency of radioactive silver from the oxide injected subcutaneously to be retained by the injected area with only a minor portion of material to be distributed into distant tissues and organs. However, the radiosilver injected into subcutaneous tissue on the periphery of a subcutaneous tumor (Fig. 2) was shown to be retained not only by the injected area but in a still greater proportion, by tumor tissue; the same material injected intraperitoneally or intrapleurally was retained within the cavity and mainly within tumor implants and exudate containing tumor cells (Fig. 4 and 6). Serous cavities of normal mice have equally retained the injected radioactive material but stored it mainly in the liver and other organs (Fig. 3 and 5). Geiger counts (Table I) provided interpretation for the data supplied by autoradiography: it appears from Table I that in normal mice (Series I) the subcutaneously injected radioactive silver oxide was transported by the blood stream into liver and spleen with a major portion of the material remaining in the

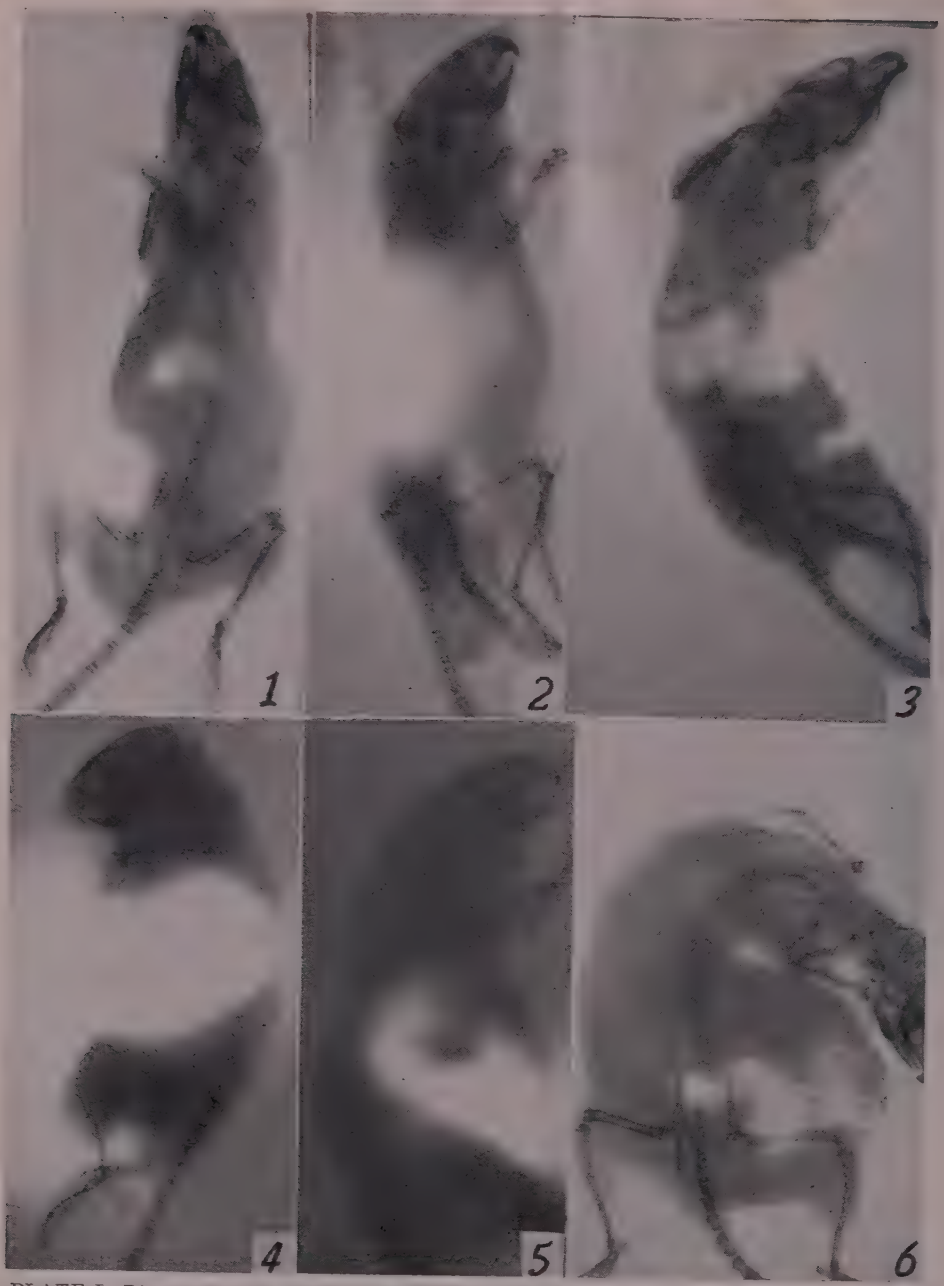

PLATE I. Distribution of $Ag^{111}O$ inj. by various routes into normal and tumor-bearing mice. (Autoradiographs.)

FIG. 1. Inj. into subcut. abdominal tissue of normal mice.
FIG. 2. Inj. into periphery of tumor on back.
FIG. 3. Inj. into normal pleural cavity.
FIG. 4. Inj. into pleural cavity containing malignant exudate and implants.
FIG. 5. Inj. into normal peritoneal cavity.
FIG. 6. Inj. into peritoneal cavity bearing malignant exudate and implants.

subcutaneous tissue at the injected area. However, the material injected into tumor bed and periphery (series 2, Table I) was stored in the tumor tissue in much higher proportion than in the injected subcutaneous tissue; only insignificant amounts were taken up by abdominal organs. In the pleural cavity of a normal animal (series 3) the radiosilver was stored mainly in the lung and the mediastinal nodes, but a significant portion of the material was translocated into the liver; a much larger portion of injected radioactive material was stored in the pleural cavity containing malignant growth (series 4), the storage taking place in the first line in tumor implants and exudate. Similar difference in distribution of radioactivity was recorded in mice with or without peritoneal malignant growth (series 5 and 6).

Microscopically, particles presumably of silver oxide were found inside macrophages of malignant exudates (pleural and peritoneal) of tumor tissue, but also abundantly as free particles in the exudate and tissue fluids; many lymphatics in the injected area of subcutaneous tissue were filled up with the material.

Discussion. Distribution of our radioactive material in normal mice reveals the role of tissue spaces, lymphatics and macrophagic elements in the storage of silver oxide particles; a large portion of material was found in organs rich in reticulo-endothelial elements (liver, spleen, lungs) and another portion was retained in the injected area, in particular after subcutaneous injection, presumably as a result of clogging of lymphatics. Tumors in the vicinity of an injected subcutaneous area drained the injected area by absorbing the particles into macrophages and interspaces of malignant tissue, thus decreasing the deposit at the site of injection and the output into the blood stream.

The competition between subcutaneous tumors and their hosts' tissues for peritumorally injected radioisotopes has been described in our report on the effect of radioactive Yttrium ($Y^{90}Cl^3$) in tumor bearing animals (3). The intracavitary retention of particulate isotopes and the role of macrophages in storage of their particles has been described for radiogold (Au^{198}) by Goldie, West *et al.* (4,5,6) and for radioactive chromic phosphate ($CrP^{32}O_4$) by Gabrielli(7) and by McCormick, Jaffe and Sied(8); the topical localization of radioactive Yttrium in the muscle and the cavities with only insignificant translocation into organs was described by Lahr, Olsen, Gleason and Tabern(9). Our present investigation adds an Ag^{111} particulate compound to the group of radioisotopes suitable for topical application. It should be noted also that Ag^{111} shares with the Au^{198}, P^{32} and Y^{90} the advantage of being short lived (half life 7.5 days) and with the latter two isotopes an additional advantage of being a pure beta (short wave) emitter, thus more suitable for selective topical application than isotopes with some proportion of gamma (long wave) irradiation which is liable to exert distant action on hematopoietic organs.

Summary and conclusions. (1) Radioactive silver (Ag^{111}) was injected in the form of the insoluble oxide into subcutaneous tissue and serous cavities of normal and tumor bearing (Sarcoma 180, Carcinoma C_3H/BA, Sarcoma McGhee, Carcinoma EO771) mice, in doses of 0.1 or 0.2 millicurie. Twenty-four hours later the animals were sacrificed; in each series some mice were used for autoradiography and others for testing radioactivity of their tissues and organs with Geiger counter. (2) Subcutaneously injected $(Ag^{111})_2O$ is stored in normal mice mainly in the injected area and in smaller proportion by liver and spleen; in subcutaneous tumor bearing mice, the major portion of silver oxide injected in vicinity of the malignant growth is taken up by malignant tissue. (3) $(Ag^{111})_2O$ injected into the normal pleural cavity is stored mainly by lungs and liver, and in tumor bearing cavity—by malignant exudate and implants. Similar difference in distribution was recorded for normal and tumor bearing peritoneal cavities. (4) It was concluded that radioactive $(Ag^{111})_2O$ deposited subcutaneously or inside serous cavities is drained by tissue or exudate fluid into adjacent malignant tissue where it is stored by intercellular fluid and by macro-

phagic elements.

1. West, H. D., Johnson, A. P., and Johnson, C. W., *J. Lab. and Clin. Med.*, 1949, v34, 1376.

2. West, H. D., Elliott, R. R., Johnson, A. P., and Johnson, C. W., *Am. J. Roentgenology*, 1950, v64, 871.

3. Goldie, H., and West, H. D., to be published in *Cancer Research*, 1956.

4. Goldie, H., and Hahn, P. F., PROC. SOC. EXP. BIOL. AND MED., 1950, v74, 634.

5. Goldie, H., Calvin, L. B., Nash, H., and Hahn, P. F., *ibid.*, 1951, v76, 480.

6. Goldie, H., West, H. D., Jeffries, B. R., and Butler, C. H., *ibid.*, 1952, v80, 327.

7. Gabrielli, E. R., *Acta Physiol. Scandinav.*, 1951, v23, 283.

8. McCormick, J. B., Mille, G., Jaffe, B., and Sied, L., *Arch. Path.*, 1954, v48, 187.

9. Lahr, T. N., Olsen, R., Gleason, G. I., and Tabern, D. L., *J. Lab. and Clin. Med.*, 1955, v45, 68.

Received April 10, 1956. P.S.E.B.M., 1956, v92.

Distribution of Glucose in Blood of the Chicken. (22404)

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The whole blood sugar value for the domestic chicken varies over a wide range(1). A portion of this variation has been attributed to a sex difference. It has been reported(2) that immature fowls of both sexes and the mature hen have a similar glycemic level which is significantly higher than that of the adult male. Orchidectomy increases the whole blood sugar value to within the range of the hen(3). In a comparative study(4) with normal and testosterone propionate injected laying hens it was observed that androgen depressed the blood sugar level significantly. A sex variation in red cell volumes, counts, and hemoglobin values has also been reported for this species(5,6). Immature fowls of both sexes and the mature hen have a lower packed-cell volume, red cell count and hemoglobin percentage than the adult male. Coincident with maturation the cockerel shows a steady increase in packed-cell volume. The mature value represents an increase of approximately one-third. Sinistral poulards also have a high red blood cell value, whereas bilaterally ovariectomzied hens remain within the normal range for the hen(6). Androgen injection into immature chickens and into adult laying hens results in a marked and fairly rapid increase in red cell volume, red cell count, and hemoglobin level, all of which may attain the normal male adult range. Castration in the male results in values which do not significantly vary from those of the hen(7). This discussion suggests an intimate relationship between whole blood sugar levels and blood cell concentration. This association is supported by reports of an unequal plasma-cell glucose partition. One such report(8) for the hen indicates only 16.3% of the glucose in the circulating cells. Similar findings have been recorded in the pigeon(9). Little or no glucose has been found in the cells of swine, dog, cat, rat, guinea pig, and human(10,11).

It was decided to study plasma glucose levels in the chicken and to test the hypothesis that the sex variation reported for whole blood glucose values is a direct function of the cell concentration, and further that the androgen hypoglycemic effect is likewise a function of its red-cell-increasing capacity.

Materials and methods. Nineteen White Leghorn laying hens were used. Feed and water were offered *ad libitum*. Fifteen to 30 ml blood samples were taken in the morning after a 16-hour fast by heart puncture with a heparin-wetted syringe. The plasma was separated within 15 minutes. Packed-cell volumes were determined with Wintrobe tubes spun at 2500 rpm for 30 minutes. Since an error of approximately 10% is incurred by this low speed technic, high speed centrifuga-

TABLE I. Whole Blood and Plasma Glucose.

Group	Animals	Packed-cell vol	Whole blood, mg % glucose	Plasma, mg % glucose	% glucose in plasma	Calculated plasma, mg % glucose
Laying hens	12	30.7 ± .598*	172.3 ± 2.95	233.1 ± 4.38	93.8 ± .329	247.3 ± 4.51
Testosterone-inj. hens	7	40.3 ± 1.18	151.4 ± 2.22	225.0 ± 6.66	88.5 ± .640	254.6 ± 7.12

* Stand. error of mean.

tion was subsequently employed and correction factors were calculated. The force applied was above 6000 G. This procedure substantially reduces the inherent error in the method. Preparation of the filtrate and titration of the sugar was done by the Somogyi Shaffer-Hartman method(12). Seven of the hens were injected intramuscularly every other day with 5 mg of testosterone propionate* in one ml of peanut oil for a total dose of 35 or 40 mg.

Results. The results in Table I show that the whole blood sugar level of the hens injected with testosterone propionate contains 20.9 mg% less glucose than the control laying hens. This difference is significant† at the one per cent level. There was no significant difference in the plasma glucose levels. The packed-cell volume of the injected hens is approximately one-third higher than that of the control hens.

Discussion. The whole blood sugar and packed cell values obtained agree with the reports describing a sex difference for the chicken. The mature hens showed a whole blood sugar value which was 12% higher than the androgen injected chickens. The injected hens showed a marked increase in circulating cells. But in both groups there was no significant difference between the plasma sugar levels. By subtracting the sugar referable to the plasma from the whole blood value, it may be seen that 88.5 to 93.8% of the circulating glucose is in the plasma and only a small fraction in the cells. Even this small amount may be due to *in vitro* changes in red cell permeability since it is known that once blood is shed glucose passes from the plasma into the cells and that anticoagulants increase the glucose permeability of the cells. Even with the use of heparin there is a slow passage of sugar into the cells. From limited trials it was observed that after 30 minutes approximately 85 per cent of the sugar remained in the plasma. In this respect it would appear that chicken blood resembles swine blood in which no transfer is observed after six hours as opposed to human blood in which passage begins immediately, is 50% complete within 10 minutes and completely equilibrated in 90 minutes(10,11).

The plasma-cell glucose partition observed in the chicken, coupled with the large difference in circulating blood cells found in the adults of the species, would explain the sex variation which has been described. As the cell concentration increases, the glucose in the whole blood is diluted. Whereas androgen has been reported to have a hypoglycemic effect, the above results show that androgen increases the number of circulating red cells but does not change the plasma glucose level.

In view of this plasma-cell partition, plasma glucose values are also shown in Table I as calculated plasma values. These were obtained by dividing the whole blood sugar value by the plasma volume percentage. This procedure circumvents the *in vitro* modifying factors by placing no restrictions on the use of anticoagulant nor by requiring plasma separation. Only whole blood and packed-cell volume determinations are needed.

Summary. Plasma rather than whole blood transports most or all of the glucose in the chicken. In view of variations in red cell concentration in this species, a measurement of plasma sugar can often be a more useful evaluation of circulating sugar concentration. The mature male and androgen injected females, which show a lower blood sugar level than immature fowls and laying hens, show

* Generously supplied by Schering Corp., Bloomfield, N. J.

† Students t applied.

no significant variation in sugar concentration of the plasma. It would therefore appear that the sex difference in blood glucose values reported for the chicken is a manifestation of variations in cell concentration. Androgen alters the whole blood sugar level by its cell-increasing action rather than by a primary action on sugar concentration.

1. Batt, H. T., *Carbohydrate metabolism in the domestic fowl. I—Variations in the fasting blood sugar level.* Thesis, Cornell University, 1938.

2. Rogemont, L., *Comp. Rend. Soc. Biol.*, 1930, v104, 154.

3. Sturkie, P. D., *Avian Physiology*, Comstock Publishing Co., Ithaca, New York, 1954.

4. ———, *Endocrinol.*, 1955, v56, 575.

5. Dukes, H. H., and Schwarte, L. H., *Am. J. Physiol.*, 1931, v96, 89.

6. Juhn, M., and Domm, L. V., *ibid.*, 1930, v94, 656.

7. Domm, L. V., and Taber, E., *Physiol. Zool.*, 1946, v19, 258.

8. Bendella, A., *Wiener Tierärztliche Monatsschrift*, 1942, v28, 44. (Abstract in *Vet. Bull.*, 1943, v13, 415.)

9. Andreen-Svedberg, A., *Skand. Arch. Physiol.*, 1933, v66, 113.

10. Olmstead, J. M. D., *Am. J. Physiol.*, 1935, v111, 551.

11. ———, *ibid.*, 1935, v114, 448.

12. Somogyi, M., *J. Biol. Chem.*, 1945, v160, 69.

Received April 11, 1956. P.S.E.B.M., 1956, v92.

Low Molecular Weight Hexosamine-Containing Compounds in Urine.*† (22405)

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Hexosamines‡ are important components of connective tissue, synovial fluid, plasma and urine, and are frequently increased in amount in connective tissue diseases such as rheumatic fever, rheumatoid arthritis and disseminated lupus erythematosus(1-3). Hexosamines in the body are, almost exclusively, constituents of high molecular weight compounds (*e.g.* mucopolysaccharides, mucoproteins). They are not known to exist in a free state in nature(4) and even dialyzable hexosamine-containing compounds have not heretofore been demonstrated in body fluids and tissues.

It has been recently established by several investigators that a large non-dialyzable hexosamine fraction exists in normal urine(3,5,6). The development of an ion exchange method for the isolation of hexosamines(7) has made it possible to study quantitatively all hexosamine compounds in urine. During the course of such a study it became evident that a portion of the total urine hexosamine was freely dialyzable(3). Data are presented here which indicate that these substances form a relatively large pool of heretofore unknown low molecular weight hexosamine-containing compounds.

Methods. Urine specimens were collected for 24 hours and either studied directly or frozen until ready for analysis. Dialysis of urine was carried out at 4°C in large volumes of distilled water (1:50). The water was changed 2 times at 24 hour intervals. The difference between the concentration of hexosamine in the bag before and after dialysis (with appropriate volume corrections) was used to calculate the per cent of dialyzable hexosamine-containing compounds in urine. After acid hydrolysis and subsequent isolation

* Investigations supported by grants from Gustavus and Louise Pfeiffer Research Foundation and Helen Hay Whitney Foundation.

† We wish to thank Drs. Neil Lebhar and Harry Trambert for assistance in obtaining these specimens and Miss Dorothy Skoglund for valuable technical assistance.

‡ Naturally occurring hexosamines are glucosamine and galactosamine. The method used here does not distinguish between these two amino sugars.

TABLE I. Urinary Excretion of Hexosamine in a Variety of Clinical Conditions.*

Diagnosis	No. observations	Hexosamine		
		Total, mg/24 hr	Dialyzable fraction	
			mg/24 hr	% of total
Myxedema—Case 1	5	52 ± 5	16 ± 3	33 ± 6
Idem 2	3	53 ± 3	20 ± 4	37 ± 5
Cholecystectomy, pre-op.	1	129	54	42
" 2 days post-op.	1	303	137	45
" 13 " "	1	106	45	42
Pregnancy, 3rd trimester	13	159 ±24	67 ±13	42 ± 5
Nephrosis	1	508	89	17
Normal adults	8	79 ± 9	37 ± 4	47 ± 5

* Stand. dev. $= \pm \sqrt{\frac{\Sigma x^2}{(n-1)}}$.

on Dowex-50, hexosamines were determined using a modification of the method of Elson and Morgan(7). Further studies on dialyzable hexosamine compounds were carried out on a lyophilized dialyzate of 20 liters of pooled normal male urine.§

Results. The relatively large amounts of low molecular weight hexosamine-containing compounds excreted in a variety of clinical conditions are shown in Table I. Normal adults excreted 79 mg of hexosamine per 24 hours, of which 47% was dialyzable. Myxedema, a condition known to be associated with low excretion of total hexosamine(3,8), also demonstrated a low excretion of dialyzable hexosamine. On the other hand, conditions known to be associated with increased excretion of total hexosamine (surgery(3), pregnancy(9)) demonstrated increases in the dialyzable fraction. This was particularly striking on the second post-operative day in the patient who had a cholecystectomy for obstructive jaundice, and a series of pregnancies studied in the third trimester. In both instances the percentage of dialyzable hexosamine excreted remained normal. In a case of juvenile nephrosis the total hexosamine excretion was markedly increased. This was due in large part to the presence of non-dialyzable hexosamine-containing plasma proteins.

Discussion. Hexosamines are present abundantly in many body tissues and fluids (4,7) in the form of mucoproteins and mucopolysaccharides. Little is known concerning the intermediary metabolism of these high molecular weight compounds. Mucopolysaccharides, when treated enzymatically (hyaluronidase, glucuronidase) can be broken down *in vitro* to low molecular weight disaccharides and oligosaccharides. The dialyzable hexosamine-containing compounds demonstrated here may represent fragments of these high molecular weight compounds.

Since these low molecular weight hexosamine-containing compounds represent only about 0.002% of the total urinary solids, their identification will depend primarily on chemical isolation methods. Preliminary gradient elution studies on cellulose columns have demonstrated at least three distinct low molecular weight hexosamine fractions(10).

Summary. Relatively large amounts of low molecular weight hexosamine-containing compounds were shown to be present in urine. The excretion rate varied from 16 mg/24 hours (myxedema) to 137 mg/24 hours (surgical stress) as compared with a normal mean excretion rate of 37 mg/24 hours.

§ Kindly prepared by Dr. John Keresztesy of the National Institute of Arthritis and Metabolic Diseases.

1. Consden, R., Glynn, L. E., and Stanier, W. M., *Biochem. J.*, (London), 1953, v55, 249.

2. Boas, N. F., and Soffer, L. J., *J. Clin. Endocrinology*, 1951, v11, 39.

3. Boas, N. F., Bollet, A. J., and Bunim, J. J., *J. Clin. Invest.*, 1955, v34, 782.

4. Boas, N. F., and Foley, J. B., PROC. SOC. EXP. BIOL. AND MED., 1955, v88, 454.

5. Hammerman, D., and Hatch, F. T., *ibid.*, 1955, v89, 279.

6. King, J. S. Jr., and Hyder, N., *ibid.*, 1955, v89, 342.

7. Boas, N. F., *J. Biol. Chem.*, 1953, v204, 553.

8. Weiner, R., *et al.*, *J. Clin. Endocrinol. and Metab.*, 1955, v15, 1131.

9. Boas, N. F., submitted for publication.

10. ———, to be published.

Received April 12, 1956. P.S.E.B.M., 1956, v92.

Unesterified Fatty Acids and Lipid Transport in Dogs.* (22406)

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This study was undertaken to investigate the relative importance of two lipid transport mechanisms, lipoproteins and unesterified fatty acids.

Materials and methods. Alimentary lipemia was produced in male, mongrel dogs by feeding 40 ml of cod liver oil. Blood samples were oxalated, promptly refrigerated, and centrifuged in the cold. Unesterified fatty acids were determined by the method of Davis(1) as modified by Grossman(2). Esterified fatty acids were estimated by the procedure of Stern and Shapiro(3). The sugar content of the plasma was determined by the method of Somogyi(4) with Nelson's colorimetric adaptation(5). All analyses were in duplicate.

Results. During absorption, most fats are transported in the blood stream as neutral lipids. It seemed important to know whether under these conditions the dog also uses the other common transport mechanism, unesterified fatty acids. Fig. 1 presents one of the experiments in which esterified and unesterified fatty acids were determined simultaneously during fat absorption. Both esterified and unesterified fatty acids increase in concentration, the latter usually somewhat more slowly. The fasting dog (Fig. 1) fails to show these changes.

It has been shown(6-8) that the injection of heparin raises the level of unesterified fatty acids in the blood. We undertook to learn whether protamine reverses this action. Fig. 2 shows the rise of the unesterified fatty-acid level following the injection of heparin into lipemic dogs, the appearance of clearing factor,† and the lowering of the esterified fatty-acid level of the plasma.‡ All 3 effects can be detected in the blood from the femoral artery and vein, right side of the heart, hepatic, portal, renal, and jugular veins. The administration of protamine to these animals, subsequent to heparin, has the opposite effect, a decrease in the unesterified and a rise in the esterified fatty-acid levels and the disappearance of clearing activity from the plasma. It can also be seen that the complete disappearance of clearing activity from the plasma does not occur immediately after the injection of protamine. A definite, but decreased, clearing activity persists for 2 to 3 minutes. This suggests that the *in vivo* inactivation of clearing factor by protamine may be more complicated than a simple binding of heparin. These experiments imply that the action of heparin and of protamine involves both lipid transport mechanisms, esterified and unesterified fatty acids.

Since unesterified fatty acid transport is probably of secondary importance during fat absorption when lipids of exogenous origin travel in the blood stream, it seemed important to investigate conditions under which endogenous lipids are transported in the blood.

* This investigation was supported by research grant (H-2005) from National Heart Institute, National Institutes of Health.

† These effects can also be demonstrated in nonlipemic dogs and in dogs fasting for 2 to 3 days.

‡ "Clearing activity of the plasma," as shown in the Figure, is the clearing in optical density units of 1 ml of a cottonseed-oil emulsion by 0.5 ml of plasma after 5 hours' incubation at room temperature.

One such condition is prolonged starvation. Fig. 3 is a representative experiment in which 48 to 72 hours of starvation caused a marked elevation of the unesterified fatty-acid level

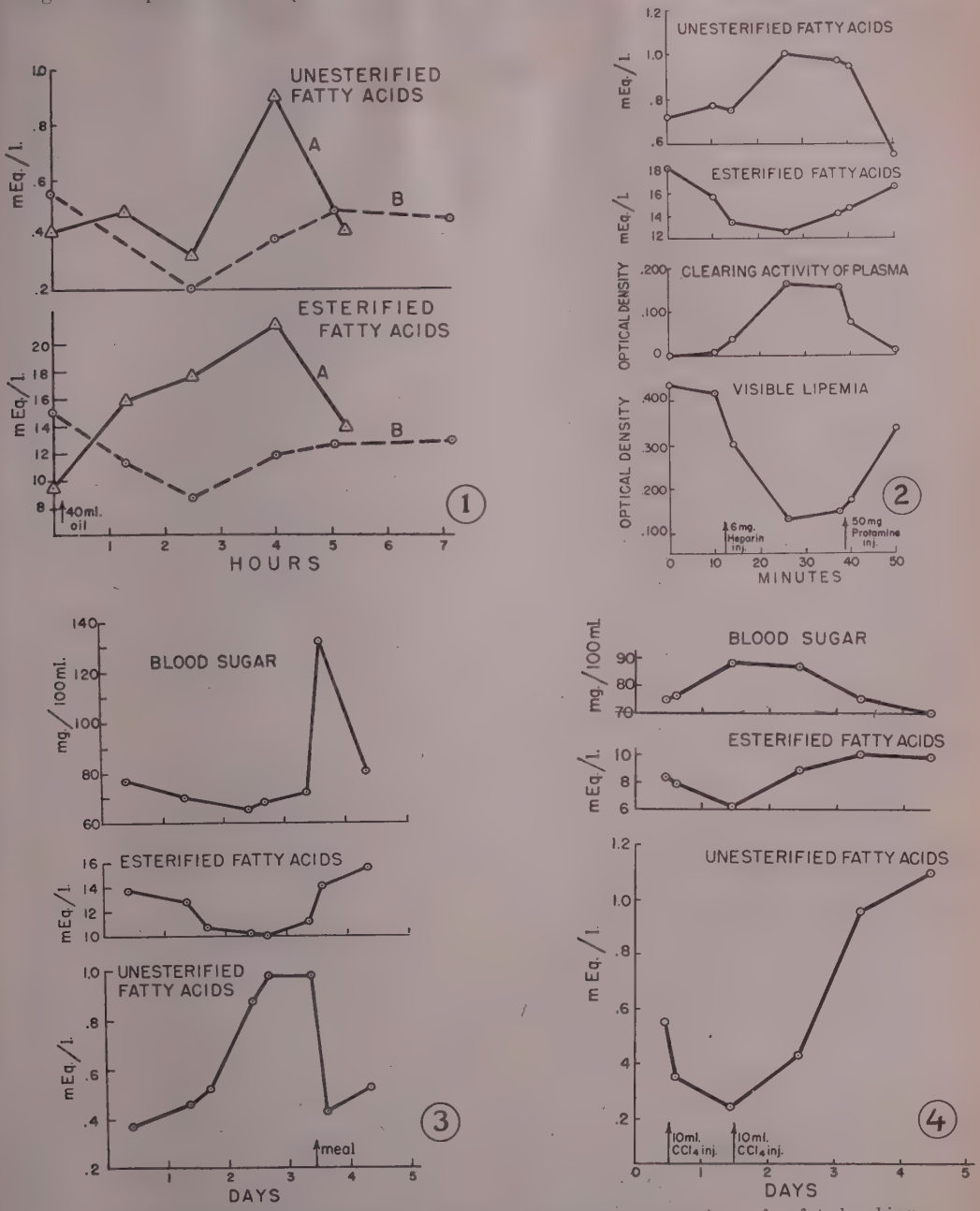

FIG. 1. Changes in unesterified and esterified fatty-acid concentrations of a fat-absorbing (A) and a fasting (B) dog.

FIG. 2. Effects of heparin and protamine in a lipemic dog.

FIG. 3. Sugar, esterified, and unesterified fatty-acid levels in plasma of a fasting dog.

FIG. 4. Sugar and fatty-acid concentration in plasma of a dog injected with CCl_4.

of the plasma, a slight decrease in the amount of the esterified fatty acid, and a very slight hypoglycemia. After feeding, all returned to normal.

Acute carbon tetrachloride poisoning was selected as another condition accompanied by considerable endogenous fat transport. The animals received injections of 10 ml of CCl_4 subcutaneously on two consecutive days, which resulted in an initial drop followed by a marked increase in the unesterified fatty-acid level of the plasma. A small increase in the amount of esterified fatty acids also occurred. Fig. 4 is a representative experiment. There was histologic evidence of typical acute lesions in the liver.

Discussion. During alimentary lipemia, both esterified fats and unesterified fatty acids increase. Conditions may be different in animals absorbing a mixed meal rather than only lipids. Dole(9) and Gordon and Cherkes(10) observed that ingestion of carbohydrates lowers the plasma unesterified fatty-acid level. This is also suggested by our occasional observation that dogs may have very low unesterified fatty-acid levels after the ingestion of a complete meal even though lipemia occurs. Results of the present investigations on the increase in unesterified fatty acids during lipemia are in accord with Grossman's findings for rats and humans(2,11).

During starvation, the total plasma lipid does not change in the dog as it does in some other species(12,13). It has long been suspected that the partition of fats changes under these circumstances(12). Our results show that fasting dogs transport fat primarily as unesterified fatty acids. In dogs poisoned with CCl_4 as in rats(14) a similar transport seems to occur.

An arterio-venous difference in unesterified fatty acids occurred in the majority of animals in the present studies. The lack of a greater consistency in the finding might reflect dynamic changes in unesterified fatty acids, especially of fat-absorbing animals.

These investigations, together with others in progress, indicate that when lipids are used as readily available energy source or are transported in the course of other metabolic functions, a portion of the lipid is transported and utilized as unesterified fatty acids. Alternatively, the esterified fats may undergo lipolysis followed by use in this form. In either case, clearing factor or a similar enzyme may play a decisive role in the metabolic channeling of lipids.

Summary. Plasma neutral fats and unesterified fatty acids were investigated in different conditions of increased lipid transport in the dog. During fat absorption, in addition to the increase in neutral fats, unesterified fatty acid also increases. Heparin and protamine influence both transport mechanisms in the post absorptive state, the former increasing the unesterified fatty acids and decreasing the esterified fatty acids; the latter opposing this effect. Starvation for from 48 to 72 hours and acute CCl_4 poisoning raise the unesterified fatty-acid level markedly. The results indicate the metabolic importance of unesterified fatty acids in lipid transport and the possible role of clearing factor in regulating lipid metabolism.

1. Davis, B. D., *Arch. Biochem.*, 1947, v15, 351.

2. Grossman, M. I., Palm, L., Becker, G. H., and Moeller, H. C., PROC. SOC. EXP. BIOL. AND MED., 1954, v87, 312.

3. Stern, I., and Shapiro, B., *J. Clin. Path.*, 1953, v6, 158.

4. Somogyi, M., *J. Biol. Chem.*, 1945, v160, 61, 69.

5. Nelson, N., *ibid.*, 1944, v153, 375.

6. Shore, B., Nichols, A. V., and Freeman, N. K., PROC. SOC. EXP. BIOL. AND MED., 1953, v83, 216.

7. Robinson, D. S., and French, J. E., *Quart. J. Exp. Physiol.*, 1953, v38, 233.

8. Brown, R. K., Boyle, E., and Anfinsen, C. B., *J. Biol. Chem.*, 1953, v204, 423.

9. Dole, V. P., *J. Clin. Invest.*, 1956, v35, 150.

10. Gordon, R. S., and Cherkes, A., *ibid.*, 1956, v35, 206.

11. Grossman, M. I., Moeller, H. C., and Palm, L., PROC. SOC. EXP. BIOL. AND MED., 1955, v90, 106.

12. Mansfeld, G., *Pflüger's Arch. f. d. ges. Physiol.*, 1909, v129, 46.

13. Deuel, H. J., *Lipids. Their Chemistry and Biochemistry*, Interscience Publishers Inc., New York, 1955, v2, 420.

14. Selden, G. L., and Westphal, U., PROC. SOC. EXP. BIOL. AND MED., 1955, v89, 159.

Received April 13, 1956. P.S.E.B.M., 1956, v92.

Respiratory Metabolism of Mammalian Eggs.* (22407)

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Little is known of the metabolic mechanisms of mammalian eggs although biochemical investigation of this tissue would seem to be of fundamental importance in the study of development and differentiation. Extensive investigations have been carried out on the eggs of several other species mainly marine animals. During the course of mammalian evolution, egg production has been reduced in number and confined to a specific phase of the sexual cycle. Consequently, metabolic studies on mammalian eggs have been very much restricted. Smith and Kleiber(1) reported that the oxygen consumption of rabbit eggs is greater than that of other eggs of similar size. Fridhandler *et al.*(2) have shown that there were little or no differences between the O_2 uptake of fertilized, unfertilized and activated (capable of subsequent cleavage) unfertilized rabbit eggs and the O_2 consumption of unfertilized ova was not depressed after remaining *in vivo* up to 57 hr *in situ*.

This paper deals with the changes in respiratory rate of rabbit eggs during development. Oxygen uptake of the early stages was measured in the Cartesian diver while that of the later stages was measured in the Warburg apparatus.

Materials and methods. Virgin rabbits weighing from 7 to 8 lbs were purchased from Rockland Farms. They were superovulated using sheep pituitary extract(3). The animals were sacrificed 0.5, 1.5, 2.5, 3.5, 5.5 and 6.5 days post-coitum. The tubes or uteri were flushed with Ca^{++}-free Krebs-Ringer phosphate pH 7.4 containing 0.1% glucose (4) "RPG." Bovine hyaluronidase (Wyeth) was used to separate the ova from any attached cumulus cells. In some cases the blastocysts were punctured and the blastocyst fluid washed out and in other cases the blastocyst was punctured and the cellular mass dissected from the zona pellucida. Only healthy ova, morulae and blastocysts were transferred to freshly prepared RPG. The respiratory rates of intact ova, morulae and 4-day-old blastocysts and punctured, dissected 5- and 6-day-old blastocysts were measured in the Cartesian diver. The Warburg respirometer was used to measure respiration in intact and punctured 6-day-old blastocysts. The Cartesian diver has been described by Linderstrøm-Lang(5,6) and Boell *et al.*(7) and Holter(8). It is capable of measuring gas exchange of less than one mμl. The ova, morulae or punctured blastocysts were pipetted into the divers using a braking pipette(8). The volume of the medium ranged from 0.7 to 1.0 μl whilst the volume of the gas space ranged from 5 to 15 μl and each diver contained 7 to 15 ova or one dissected blastocyst. Thirty to fifty 6-day-old intact or punctured blastocysts were transferred to Warburg flasks (fluid volume 1 ml) using freshly prepared RPG as medium. In all experiments, air was the gas phase and the temperature of the water bath was 35°C.

Results. The average O_2 uptake of the fertilized rabbit ova 16 hours post-coitum was 0.61 mμl/ovum/hr. Detaching the cumulus cells from the freshly shed ova was found to be necessary, since they respire at a high rate. A mass of cumulus cells roughly equivalent in volume to one ovum consumes O_2 at the rate of 2.5 mμl/hr. Smith and Kleiber (1) reported that the O_2 uptake of the fertilized one-cell rabbit ovum was 26 mμl/ovum/hr as measured in the Cartesian diver. The difference between their value and that reported here is too great to be attributed to

* Thanks are due to Mr. William Ducharme, Jr. for technical assistance. Whole sheep pituitary powder was donated by Parke, Davis & Co. and bovine hyaluronidase by Wyeth Institute for Medical Research. This work was aided in part by research grants from Josiah Macy, Jr. Foundation and Planned Parenthood Federation of America.

† On research leave from Faculty of Agriculture, Cairo University, Cairo, Egypt.

strain, live weight or nutritional differences in the experimental material. It should be pointed out that their conditions (incubating medium, temperature and gas phase) were different from the present experiment. Dragoiu *et al.*(9) reported that the respiratory rate of cow's follicular eggs (with germinal vesicle) was 5000/mμl/egg/hr. This value probably represents the respiration of follicular cells of corona radiata predominantly, for, in the experiments cited, a single egg plus its halo of follicle cells had an average dry weight of more than 8 μg, a figure which is some 10 times larger than the estimated dry weight of a single bovine egg. Moreover in the cow's egg cumulus cells are present in the large follicles(10). In the rat, the O_2 consumption of follicular eggs and fertilized ova was 1.11 and 0.72 mμl/egg/hr respectively(11).

The average O_2 uptake of the fertilized ova 40, 65, 90 and 160 hr post-coitum was 0.51, 0.48, 2.56 and 200 mμl/hr/ovum. There was no increase in the O_2 uptake during the first 65 hr post-coitum *i.e.* up to the morula stage. There was a sudden rise in the respiratory rate at the early blastocyst stage (Fig. 1). This was definitely associated with the morphological changes which generally supervene by the 80th hr post-ovulation. If the morulae, however, failed to develop into blastocysts by the 80th hr post-ovulation, the respiratory rate remained essentially unchanged as compared with that of the one-cell stage.

In the Warburg apparatus, there was a

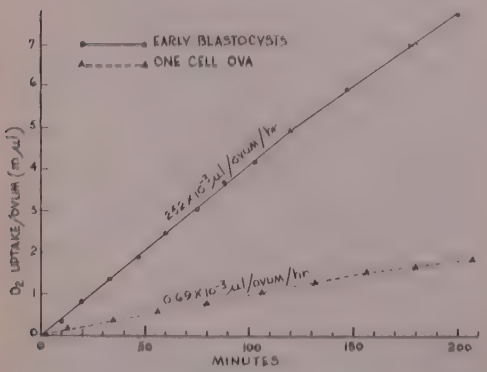

FIG. 1. O_2 consumption of one-cell ova and early blastocysts as measured in the Cartesian diver.

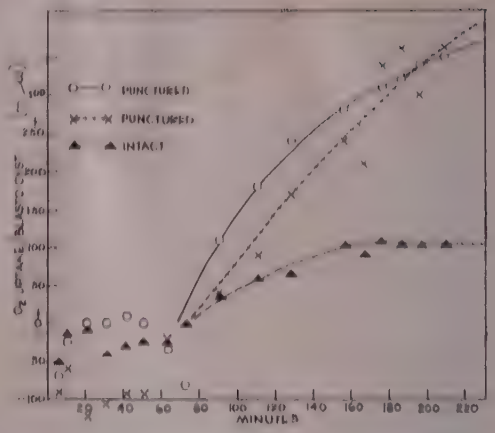

FIG. 2. O_2 consumption of rabbit blastocysts 160 hr post-coitum as measured in Warburg respirometer.

period of no apparent O_2 uptake in the first 30 minutes after the closure of all the vessels. This was followed by a simultaneous initiation of O_2 uptake in all the vessels. The rate was uniform but tended to decline with time. The initial rate was lower in the intact as compared with the punctured blastocysts (Fig. 2). In fact, in the intact blastocyst the respiration stopped entirely after 30 minutes. This is probably due to the high bicarbonate content of the blastocyst fluid which caused, in the presence of the CO_2 free atmosphere in the vessels, a rise in the pH to 9.5.

Five- and 6-day-old blastocysts were punctured, dissected from the zona pellucida, washed free of blastocyst fluid and split into 2 segments. The average O_2 uptake for the 5- and 6-day-old blastocyst was 400 and 800 mμl/blastocyst/hr respectively. The zona pellucida did not show any respiratory activity. It must be noted that the O_2 uptake was observed with no lag period in the divers in contrast with the apparent absence of O_2 uptake in the first 30 minutes in the Warburg vessels. As yet there is no apparent explanation for this difference in result.

The cellular mass of a dissected blastocyst was pipetted into a calibrated capillary tube to measure its approximate volume. Judging from this and from the diameter of the ovum the marked increase in the respiratory rate

(about 1600-fold) is not accompanied by a corresponding increase in the tissue mass. It is therefore suggested that a change in metabolism takes place at the early blastocyst stage; either an increase in the overall rate per unit of tissue mass or a shift of emphasis from one pathway to another. Boell and Nicholas(11) have suggested that the increase in respiration is intimately correlated with the increase in the volume of the egg. It must be remembered here that they worked in a narrow range of stages of development (one- to 16-cell stage). Moreover, the increase in egg volume does not necessarily represent an actual increase in dry weight. It may be mentioned here that water uptake occurs in eggs of other species during the transformation of yolk into active protoplasm(12-14) and that this process is associated with an increase in respiration.

Microscopic examination of the eggs after the respiratory measurements revealed that the morphological appearance was essentially normal. The critical test for viability by transplanting the eggs into the uteri of pseudopregnant rabbits was not performed. It has been found that the rabbit eggs do not cleave in RPG *in vitro* nor do they cleave in several synthetic media tried by Hafez and Pincus(15). Therefore the egg in RPG is not a developing organism, but a resting one and interpretation of results must be made with this in mind.

Summary. Respiration of fertilized rabbit eggs (intact; punctured or dissected) in different pre-implantation stages was measured in the Cartesian diver and Warburg apparatus. Average O_2 uptake of eggs 16, 40, 65, 90, 120, 140 and 160 hr post-coitum was 0.61, 0.51, 0.48, 2.56, 200, 400 and 800 mμl/ovum/hr respectively. The high respiratory rate of blastocysts was associated with the morphological changes yet the rate of increase was not directly proportional to cell mass increase. It is suggested that these pacts reflect a change in metabolic pathways. Intact blastocysts (in Warburg) respire at a lower rate than punctured ones due to the bicarbonate content of blastocyst fluid which raised the pH in the CO_2-free atmosphere. Cumulus cells respire at a high rate while the zona pellucida does not respire at all.

1. Smith, A. H., and Kleiber, M., *J. Cell. Comp. Physiol.*, 1950, v35, 131.

2. Fridhandler, L., Hafez, E. S. E., and Pincus, G., *Proc. IIIrd Int. Congr. Anim. Reprod.*, Cambridge, in press.

3. Pincus, G., *Anat. Rec.*, 1940, v77, 1.

4. Umbreit, W. W., Burris, R. H., and Stauffer, J. F., *Manometric Techniques and Tissue Metabolism*, Burgess, Minneapolis, 1949.

5. Linderstrøm-Lang, K. W., *Nature*, 1937, v140, 108.

6. ———, *C. R. Trav. Lab. Carlsberg Ser. Chim.*, 1943, v24, 333.

7. Boell, E. J., Needham, J., and Rogers, V., *Proc. Roy. Soc. Lond.* B, 1939, v127, 322.

8. Holter, H., *C. R. Trav. Lab. Carlsberg, Ser. Chim.*, 1943, v24, 400.

9. Dragoiu, J., Benetato, G., and Opreanu, R., *C. R. Soc. Biol.*, 1937, v126, 1044.

10. Webster, J., *Eirstock und Ei Befruchtung und Unfruchtbarkeit bei den Haustieren*, Berlin, 1921, p. 146.

11. Boell, E. J., and Nicholas, J. S., *J. Exp. Zool.*, 1948, v109, 267.

12. Gray, J., *J. Exp. Biol.*, 1926, v4, 215.

13. Dempster, W. T., *Biol. Bull.*, 1930, v58, 182.

14. ———, *J. Exp. Zool.*, 1933, v64, 495.

15. Hafez, E. S. E., and Pincus, G., in the course of publication.

Received April 13, 1956. P.S.E.B.M., 1956, v92

Experimental Mammary Gland Growth in Rabbits by Estrogen and Progesterone.*† (22408)

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The role of estrogenic hormones in stimulating growth of the duct system of experimental animals has been studied extensively in this laboratory. The mouse(4,13,14); the rat(4,16); the rabbit(11,12); the cat(10) and dog(15) all showed extended duct growth following estrogen administration. In the guinea-pig(5-14), lobulealveolar development could be stimulated with estrogens. When crude or purified preparations of progesterone became available, Turner and Frank(12) showed that combination of estrogen and progesterone would carry growth of the duct system to complete lobule-alveolar development. These observations were extended to mouse(14) and rabbit(12). The first quantitative study of amounts and proportions of these 2 ovarian hormones for optimal growth of lobule-alveolar system in the rabbit was reported by Lyons and McGinty(6) and Scharf and Lyons(7) who observed optimal synergism of the 2 hormones when 24 to 96 μg of estrone were administered with 1 mg of progesterone daily for about 25 days. This is a ratio of 1:11 to 42. In subsequent studies of amounts and proportions of these 2 hormones on growth of glands in other experimental animals, the ratio was 1 of estrogen to 1,000 or more of progesterone in mice(1,8,17), rats (1,3) and dogs(9). The great discrepancy between the ratio of estrogen to progesterone for optimum lobule-alveolar growth in the rabbit and that required by other species is of considerable importance since in larger animals such as goats and cattle, use of narrow ratios has not been successful in growing the mammary gland. With development of a more objective measurement of mammary gland growth based upon nucleic acid values (2,3) it seemed important to repeat the study of the rabbit to determine whether previous observations based upon visual observations of glands were accurate and thus to determine whether the rabbit was an exception in the narrow ratio of ovarian hormones required for mammary gland lobule-alveolar growth.

Material and methods. Normal New Zealand White male rabbits weighing 4½ to 7 lb were used. Hormones employed were estradiol benzoate and progesterone dissolved in sesame oil. The hormones were injected subcutaneously once per day for indicated period. Animals were sacrificed a day following the last administration of the hormones, skinned, and the fatty pads containing mammary glands were dissected free of lymph nodes and other tissues. All glands except the most anterior pair located in the loose skin of the neck were removed for nucleic acid determinations. Determinations of extent of mammary gland growth were primarily based on increase of mammary gland nucleic acid values, and especially upon DNA, believed to be related to cell multiplication (2,3). Mammary glands were also studied in whole mounts for visual verification of the extent of growth.

Experimental. To ascertain optimum levels of estradiol benzoate necessary to stimulate maximum extension of the duct system 6, 9, 12 and 15 μg were administered daily for twenty days to groups of 5 rabbits each. All four levels of hormone showed good duct growth with almost negligible alveolar formation. The 9 μg level was used as pretreatment dosage in the subsequent study due to the slightly higher nucleic acid values (Table I).

Groups of male rabbits were injected with 9 μg of estradiol benzoate daily for 20 days to induce duct development. Since 1 mg of progesterone daily seemed to be adequate as

* Contribution from Department of Dairy Husbandry, Mo. Agr. Exp. Sta. Journal Series No. 1614. Approved by Director.

† This investigation was supported in part by grant from American Cancer Society.

TABLE I. Nucleic Acid Values of Male Rabbit Mammary Glands following Stimulation with the Ovarian Hormones. 5 animals/series.

Treatment			DNA, mg/500 mg	PNA, mg/500 mg
μg EB		Days	DFF glands	DFF glands
0		—	3.15 ± .29	.50 ± .18
		Estrogen only		
6		20	3.47 ± .62	1.39 ± .83
9		"	3.72 ± .38	1.82 ± .52
12		"	3.56 ± .49	1.64 ± .36
15		"	3.61 ± .87	1.76 ± .97
		20 days pretreatment with estrogen		
5	+ 1 mg P	30	9.25 ± 1.60	4.00 ± 1.94
10		"	9.61 ± .97	4.20 ± 1.47
15		"	13.40 ± 1.33	7.99 ± 1.79
20		"	10.36 ± 1.38	5.31 ± 1.49
30		"	11.00 ± 1.14	5.43 ± 1.54
60		"	12.58 ± 2.93	7.70 ± 2.72
90		"	11.50 ± 1.23	6.13 ± 1.37

DFF denotes dried fat-free glands.
EB denotes estradiol benzoate; P denotes progesterone.

shown in previous studies(6), the problem to be resolved was the proper level of estrogen which would synergize with progesterone. In order, levels of 5, 10, 15, 20, 30, 60 and 90 μg of estradiol benzoate plus 1 mg of progesterone were administered daily for 30 days to groups of rabbits.

The highest nucleic acid values were obtained in glands from rabbits receiving 15 μg of estradiol benzoate plus 1 mg of progesterone. As substantiated by whole mounts of the glands, these amounts and proportions of the 2 hormones brought out full extension and development of the duct system and lobule-alveolar proliferation, which most closely approximated lobule proliferation attained during the first two-thirds of pregnancy or that developed by normal pregnancy.

The levels of 30, 60 and 90 μg of estradiol benzoate plus 1 mg of progesterone daily produced mammary glands with high nucleic acid values and generally with good lobule formation. The two lower amounts (5 and 10 μg of estradiol benzoate plus 1 mg of progesterone) produced only fair lobule development. Alveoli seemed to be in process of multiplication and the lobules were irregular and isolated. At 20 μg level daily extension of the duct system associated with lobule-alveolar system appeared greater.

Discussion. The present study using DNA content of mammary gland in conjunction with visual examination as indications of extent of growth stimulation confirms previous work indicating that the rudimentary duct system of the male rabbit can be caused to grow extensively with minimum lobule-alveolar growth following the injection of 6 to 15 μg daily of estradiol benzoate for 20 days. Our data indicated that 9 μg of estradiol benzoate daily for 20 days was optimal in the development of the duct system. Since estradiol benzoate is a more potent estrogen than estrone, these results and those of Lyons and McGinty(6) using 12 μg of estrone per day are considered in good agreement.

The most favorable level of estradiol benzoate to synergize with 1 mg of progesterone daily to obtain optimal lobule-alveolar growth was 15 μg based upon the DNA content of the glands. Smaller amounts of estradiol produced glands lower in DNA and visually inferior in lobule development. Estradiol in amounts varying from 20 to 90 μg with 1 mg of progesterone produced glands with good lobule-alveolar development. In comparison, Scharf & Lyons(7) reported that estrone in amounts from 24 to 96 μg daily synergized best with 1 mg of progesterone. Considering the difference in the biological activity of the estrogens used, the results, again, are in good agreement.

From these data, it is concluded that the rabbit differs greatly in the ratio of synergism of the 2 ovarian hormones (estrogen; progesterone) in stimulating lobule-alveolar mammary gland growth in comparison with the mouse, rat and dog. In relation to body weight of these species, the amount of estrogen required by the rabbit is relatively high in comparison to the dog (dog, 10 μg; rabbit, 15 μg) whereas progesterone requirement of the rabbit is low, 1 mg as contrasted to 0.5 mg for the mouse, 2-5 mg for the rat, and 10 mg for the dog.

The daily injection of 15 μg of estradiol benzoate and 1 mg of progesterone (ratio of 1:67) for 30 days, found to be effective for optimal lobule development in the male rabbit pretreated with 9 μg of estradiol benzoate

for 20 days has been shown to be even more effective in ovariectomized female rabbits in our laboratory as evidenced by the more pronounced overlapping of the adjacent mammary glands at their peripheries. These glands were comparable to the mammary glands of pseudo-pregnant rabbits routinely prepared preliminary to lactation studies in our laboratory.

Summary. 1. Daily injection of 9 μg of estradiol benzoate for 20 days induced extensive development of the mammary duct system of male rabbits with negligible lobule formation. 2. With male rabbits pretreated with estrogen to develop the duct system, the daily injection of 15 μg of estradiol benzoate combined with 1 mg of progesterone (synergistic ratio 1:67) induced the extensive development of the lobule-alveolar system as indicated by the DNA content and by visual examination of whole mounts of the glands. 3. These observations indicate that the optimum synergistic ratio of the ovarian hormone in lobule-alveolar development in the rabbit differs widely from the ratio of 1:1000 or more observed in the mouse, rat and dog.

1. Elliott, J. R., and Turner, C. W., *Mo. Agr. Exp. Sta. Res. Bull.*, 1938, No. 337.

2. Kirkham, W. R., and Turner, C. W., PROC. SOC. EXP. BIOL. AND MED., 1933, v33, 123.

3. ———, *ibid.*, 1934, v37, 130.

4. Lewis, A. A., and Turner, C. W., *ibid.*, 1941, v48, 439.

5. ———, *Endocrinology*, 1942, v30, 585.

6. Lyons, W. R., and McGinty, D. A., PROC. SOC. EXP. BIOL. AND MED., 1941, v48, 85.

7. Scharf, G., and Lyons, W. R., *ibid.*, 1941, v48, 86.

8. Sigel, M. B., and Turner, C. W., M. S. Thesis, Univ. of Missouri, 1934.

9. Trentin, J. J., Devita, J., and Gardner, W. U., *Anat. Rec.*, 1952, v113, 168.

10. Turner, C. W., and DeMoss, W. R., *Mo. Agr. Exp. Sta. Res. Bull.*, 1934, No. 207.

11. Turner, C. W., and Frank, A. H., *ibid.*, 1930, No. 148.

12. ———, *ibid.*, 1932, No. 174.

13. Turner, C. W., Gardner, W. U., Schultze, A. B., and Gomez, E. T., *Anat. Rec.*, 1932, v53, 277.

14. Turner, C. W., and Gomez, E. T., *Mo. Agr. Exp. Sta. Res. Bull.*, 1934, No. 206.

15. ———, *ibid.*, 1934, No. 207.

16. Turner, C. W., and Schultze, A. B., *ibid.*, 1931, No. 157.

17. Yamada, J., Nagai, J., and Naito, M., *Endocrinologia Japonica*, 1954, v1, 63.

Received April 16, 1956. P.S.E.B.M., 1956, v92.

Convulsant Effects of Semicarbazide in Epileptic Monkeys.* (22409)

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Semicarbazide (amino-urea) has been described as a potent convulsant and its action in normal monkeys has been reported(1). The present investigation was undertaken to study the comparative effects of its administration to chronically epileptic as well as normal monkeys.

Method. Semicarbazide was injected by the rapid intravenous route at intervals of one or more weeks to a group of 8 *Macaca mulatta* (3.5-6.0 kg). Five monkeys of this group had been made chronically epileptic by application of alumina cream to the brain (2), and 3 were unoperated normal controls. Each monkey was observed for periods up to 6 hours following injection. Clinical observations and electroencephalographic studies were made.

Results. Threshold convulsant dosages which provoked clinical motor seizures in the 3 normal monkeys were 80, 80, and 60 mg/kg respectively. Time of onset of clinical seizures was approximately 3 hours after intravenous injection in 2 monkeys and 4 hours in the third (60 mg/kg).

* Aided in part by Grant NIH, USPHS.

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Epileptic monkeys had threshold convulsant dosages of 10 or less, 20, 20, 30, and 30 mg/kg. Time of onset of clinical seizures varied from 2 to 3½ hours. Focal onset of clinical seizures was sometimes evident in this group. Shortening of the latency period for motor convulsions occurred in the epileptic group when suprathreshold dosages were used. Thus in epileptic monkey #757 latency was progressively reduced from 2 hours with a threshold dose of 20 mg/kg to 15 minutes with a dose of 90 mg/kg. Repeated tonic

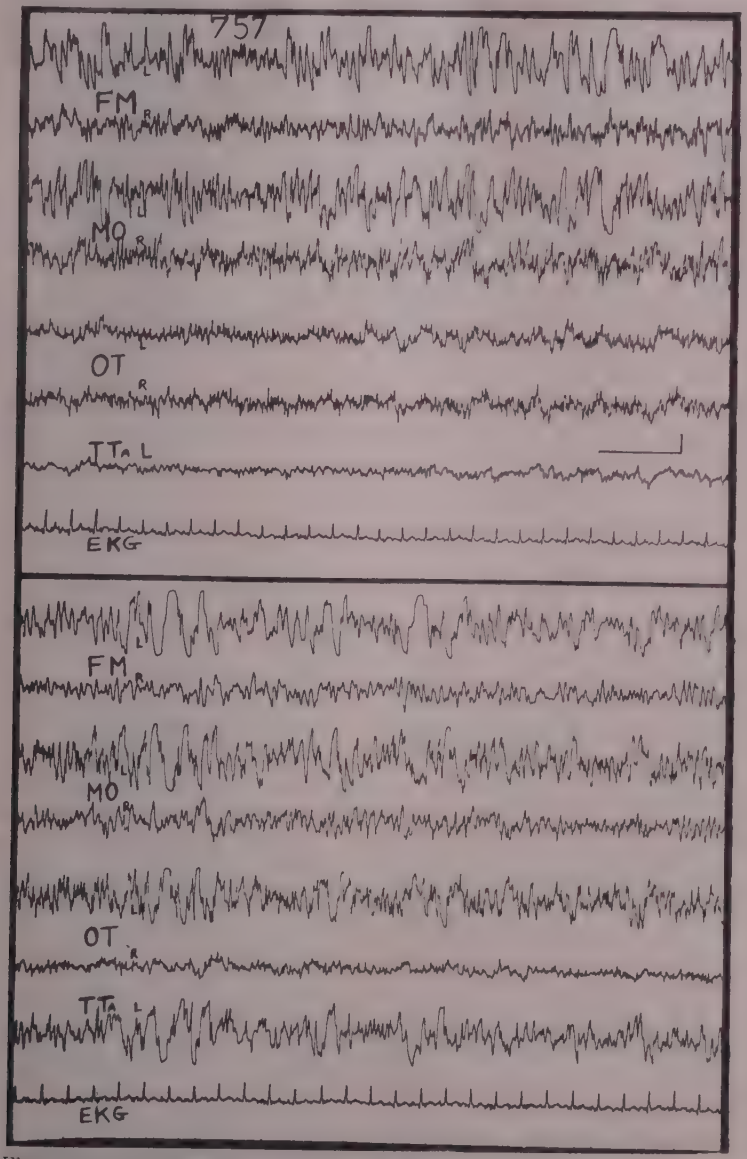

FIG. 1. Electroencephalograms before (top) and 36 min. after (bottom) intravenous inj. of 80 mg/kg semicarbazide in epileptic monkey demonstrating spread of spike, sharp, and slow waves in left cerebral hemisphere which occurred 30 sec. before clinical motor seizure. L = Left; R = Right; F = Frontal; M = Motor; O = Occipital; T = Temporal; Ta = Anterior temporal. Calibration: 1 sec. (horizontal), 50 microvolts (vertical).

clonic seizures occurred particularly with the larger doses.

Electroencephalographic changes accompanied motor seizures, and in epileptic monkeys sometimes briefly (up to ½ minute) preceded the onset of clinical seizures (Fig. 1). Significant detectable changes were otherwise not evident; increased muscle artefact potentials became more prominent after injection when the animals appeared to become more restless. Clinical seizures were accompanied by diffuse spike convulsive patterns.

Summary. (1) Threshold convulsant dosages to rapid intravenous injection of semicarbazide were significantly lower in chronically epileptic monkeys than in normal unoperated controls. (2) Clinical seizures following intravenous injection of threshold convulsant doses of semicarbazide occurred after a latent period of 2 to 3½ hours. With suprathreshold doses the latent period was diminished greatly. (3) Use of a test dose of 30 to 40 mg/kg of semicarbazide by rapid intravenous injection is suggested to distinguish epileptic from non-epileptic monkeys since clinical convulsions should be produced within 3 to 4 hours in epileptic monkeys and not in non-epileptic normals.

1. Lowell, D. J., Ross-Duggan, J. K., and Piazza, S., *J. Pharm. & Exp. Ther.*, 1952, v106, 404.

2. Kopeloff, L. M., Chusid, J. G., and Kopeloff, N., *Neurology*, 1954, v4, 218.

Received March 1, 1956. P.S.E.B.M., 1956, v92.

Histopathology of Amino Acid Deficiencies. V. Isoleucine.* (22410)

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The indispensability of isoleucine for growth in rats was demonstrated by Womack and Rose(1). Later, in experiments with dogs, several investigators showed that isoleucine was necessary for the maintenance of nitrogen balance(2), the formation of hemoglobin(3) and of plasma protein(4). The essential nature of this amino acid for human nutrition was established by Rose and his coworkers(5). Hegsted, McKibben and Stare (6) and Albanese(7) reported that the isoleucine content of human plasma protein and hemoglobin was insufficient to support normal growth in rats. Histological studies of isoleucine deficiency have not been reported for any species. The present report is the fifth in a series dealing with the histopathological effects of the total omission of an essential amino acid from the diet of rats.

Method. Young male Sprague-Dawley rats were divided into 3 groups. One group of 5 rats (normal-control) received a purified synthetic diet consisting of a mixture of 19 crystalline amino acids, vitamins, sucrose, cottonseed oil and the necessary minerals and salts as described by Rose, Oesterling and Womack (8). A second group of 15 rats (deficient) was fed the same diet, complete in every respect except for the total omission of isoleucine. The caloric value of the missing amino acid was supplied by additional sucrose. A third group of rats was pair-fed the complete diet so that the food consumption of each was no greater than that of its isoleucine-deficient mate. All rats were kept in individual cages and, with the exception of the pair-fed group, fed *ad libitum*. At the end of a 30-day experimental period, all rats, with the exception of 2 deficients, were sacrificed by decapitation without anesthesia. As rapidly as possible, the liver of each rat was removed, a small portion fixed for histological procedures, and the remainder immersed in liquid nitrogen for total glycogen determination by the method

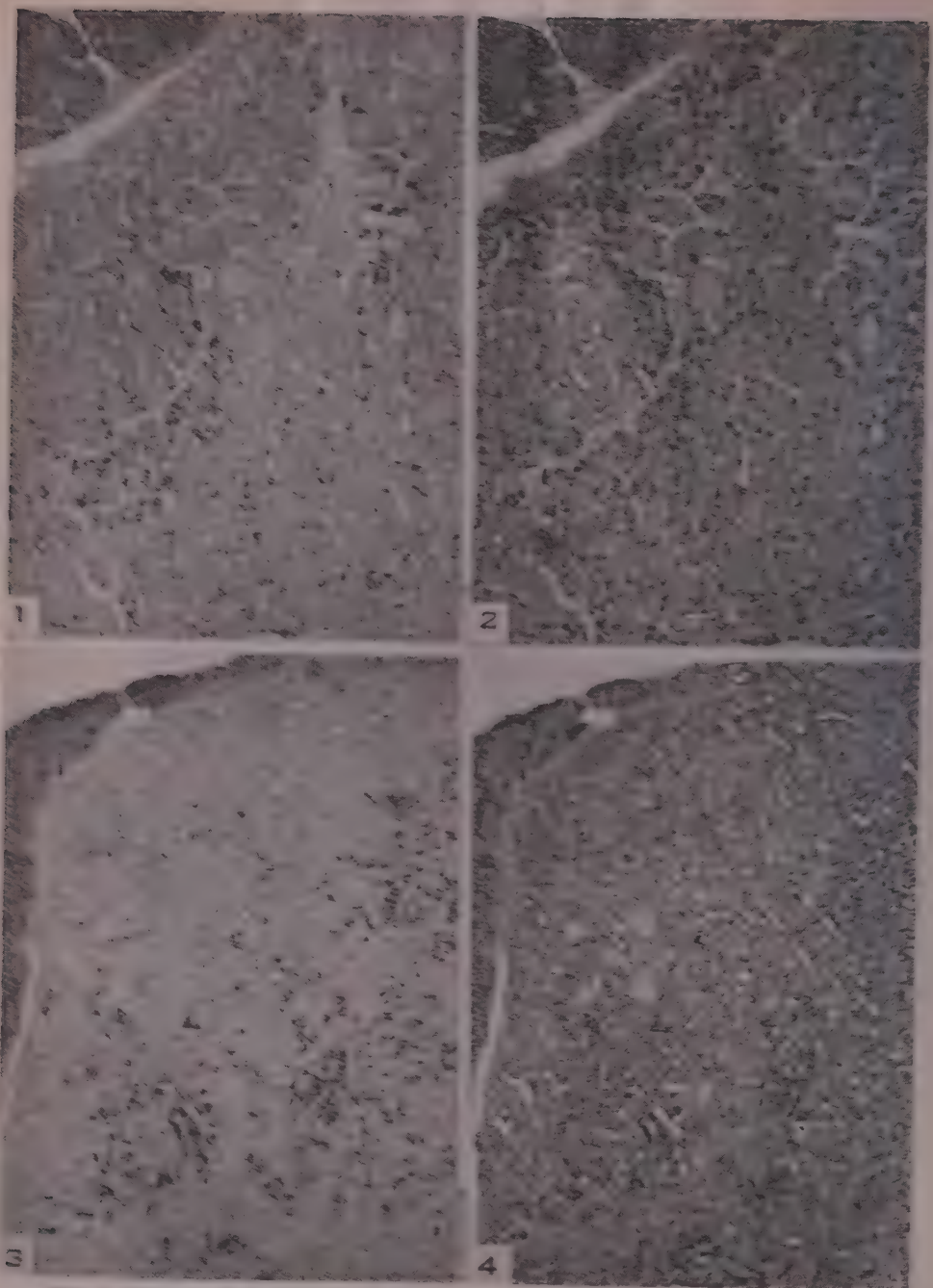
* This research was supported by grant A-289 from National Institute of Arthritis and Metabolic Diseases, U. S. Public Health Service.

TABLE I. Comparison of Body Weights and Organ Weights of Isoleucine Deficient, Pair Fed Control, Normal-Control and Recovered Rats.

Body wt (g)			Organ wt (mg)		
Initial	Final	Change	Adrenals (2)	Testes (2)	Pituitary
		Isoleucine-deficient			
46	31	−15	18.0	116.8	2.4
45	27	−18	18.2	137.8	2.4
48	31	−17	17.4	135.2	1.6
54	34	−20	17.2	151.2	2.6
50	34	−16	19.2	142.4	2.6
47	28	−19	13.2	115.2	1.4
56	34	−22	18.8	185.6	2.0
55	35	−20	15.6	148.8	1.0
51	32	−19	15.2	188.4	1.4
53	32	−21	17.4	150.4	1.8
60	38	−22	17.6	217.2	2.8
64	32	−32	17.0	173.2	1.2
55	33	−22	18.2	141.8	0.8
Mean	32.4 ± .78*		17.2 ± .44	154.2 ± 7.85	1.9 ± .18
Avg % of body wt			.053	.048	.006
		Pair-fed control			
47	45	− 2	20.4	268.4	1.4
52	41	−11	20.4	258.4	2.0
53	43	−10	24.4	297.4	2.4
49	42	− 7	20.0	516.8	2.8
50	45	− 5	22.8	546.6	2.4
55	46	− 9	18.4	373.2	3.2
52	45	− 7	18.8	546.0	
49	40	− 9	17.4	324.2	2.4
50	43	− 7	20.6	299.2	2.6
52	42	−10	19.2	373.4	2.2
43	42	− 1	19.8	532.8	2.2
46	38	− 8	21.8	239.0	2.0
51	38	−13	18.6	288.8	2.4
Mean	42.3 ± .67		20.2 ± .50	374.2 ± 31.6	2.3 ± .12
Avg % BW			.048	.089	.006
		Normal-control			
51	99	+ 48	33.2	2012.6	5.6
86	188	+102	37.5	1597.0	8.3
70	184	+114	36.3	2721.5	8.0
65	184	+119	29.4	2595.5	7.0
72	191	+119	37.6	2639.3	7.4
Mean	169.2 ± 15.7		34.8 ± 1.38	2313.2 ± 195.2	7.3 ± .42
Avg % BW			.021	1.4	.004
		Recovered			
67	210 (33)†		37.3	2673.9	7.3
68	210 (33)†		25.9	2712.9	8.0
Avg % BW			.015	1.3	.004

* Stand. error of the mean.
† Wt of these rats at end of deficient period when they were placed on recovery diet.

of Good, Kramer and Somogyi(9). Small pieces of liver, fixed in Gendre's fluid, were sectioned and stained by the Best's carmine and periodic acid-Schiff (PAS) methods for glycogen. All other tissues, except the pituitaries, were fixed in Bouin's solution and stained with hematoxylin and eosin. The pituitary bodies were fixed in Zenker-formol and stained by the aldehyde-fuchsin (AF) and periodic acid-Schiff (PAS) methods. These technics were developed by Halmi(10) and Purves and Griesbach(11,12) to demonstrate and differentiate the thyrotrophic and gonadotrophic basophils of the anterior pituitary. Employment of these methods has been described in our earlier reports(13,14). Pituitary sections were also stained with acid fuchsin for examination of the acidophils.



pituitary of a normal-control rat stained by aldehy[illegible] [illegible] AF
[illegible] ion of thyrotrophic basophils (dark cells). ×100.

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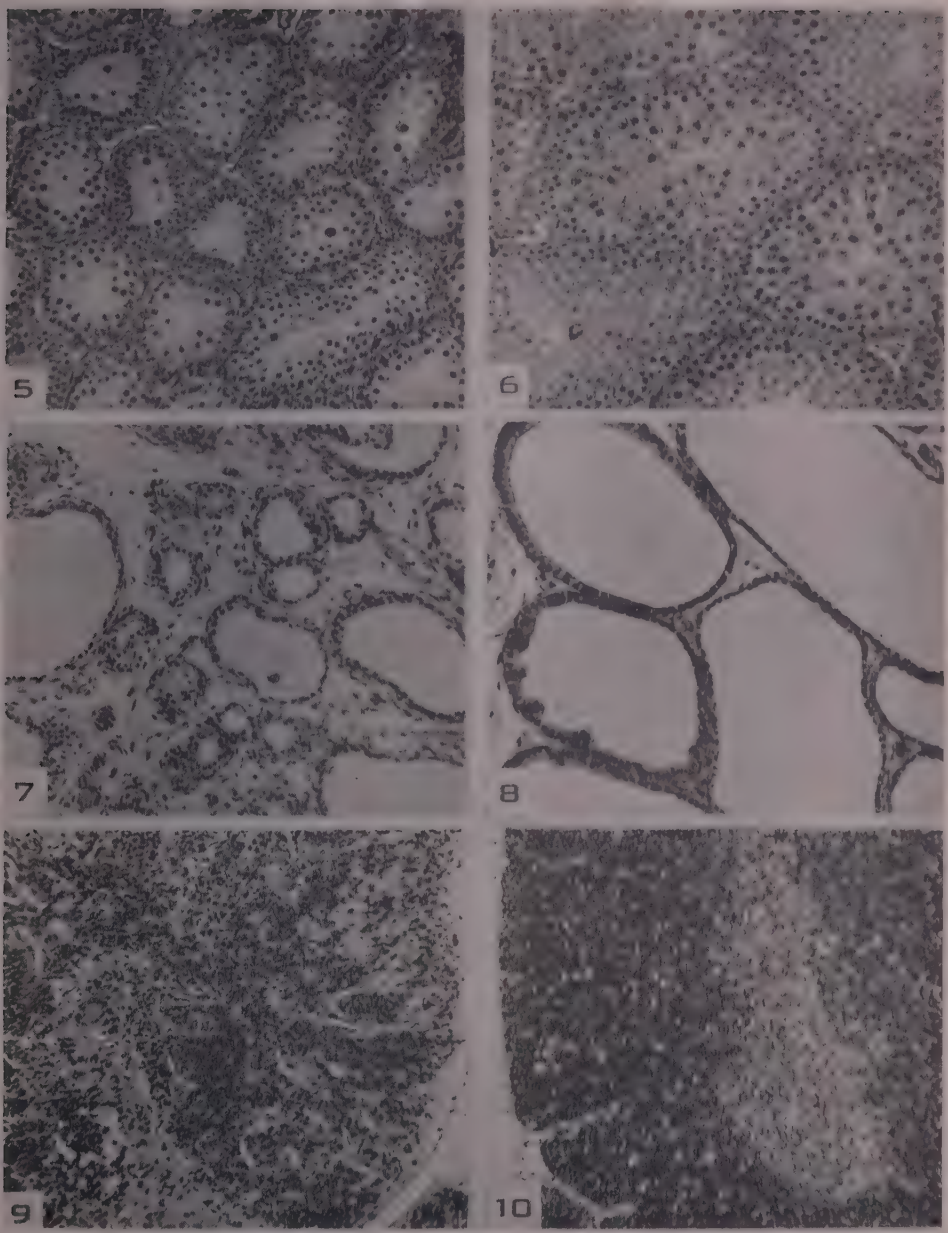

FIG. 5. Testis of an isoleucine-deficient rat. ×100.
FIG. 6. Testis of a pair-fed control rat. ×100.
FIG. 7. Prostate of an isoleucine-deficient rat. ×100.
FIG. 8. Prostate of a pair-fed control rat. ×100.
FIG. 9. Thymus of an isoleucine-deficient rat. ×100.
FIG. 10. Thymus of a pair-fed control rat. ×100.

Two isoleucine-deficient rats were not sacrificed with the others, but were placed on the complete diet (including adequate isoleucine) for a 30-day recovery period and then sacrificed.

Observations. Loss in body weight was consistently greater in the isoleucine-deficient rats than in their pair-fed mates. Table I lists the initial and final body weights as well as the weights of the adrenals, testes and pituitary of each rat. Comparison of the adrenals and pituitaries on an average percentage of body weight basis shows no significant difference between the deficient and pair-fed groups.

Histological examination of the pituitary bodies, testes and accessory sex glands showed that the total lack of isoleucine resulted in alterations which were similar to the changes observed by us in rats deprived of dietary threonine(13), histidine(14) or tryptophan (15). The pituitary thyrotrophic cells were not affected (Fig. 3), the gonadotrophic cells were completely deleted (Fig. 4) and the pituitary acidophils greatly reduced in size. The lack of gonadotrophic stimulation from the pituitary resulted in atrophy of the seminiferous tubules (Fig. 5) and testicular interstitial cells. Spermatogenesis was completely inhibited. The accessory sex glands suffered, in turn, from the lack of Leydig cell hormonal support. The prostatic epithelium was flat, atrophic and non-secretory (Fig. 7). The amount of interstitial stroma was increased; edema was slight.

In the pair-fed control rats the pituitary gonadotrophic cells, while not giving the intensely positive PAS reaction of the normal-controls, were demonstrable, although functioning at a reduced rate. This was evident by the histological appearance of the prostates and testes of these animals. The seminiferous tubules were smaller in diameter than normal but not so seriously reduced as in the deficients. Spermatogenesis lagged, but spermatozoa were being formed (Fig. 6). The prostatic epithelium was receiving some, but not total, stimulation from the testicular interstitial cells. Much of the prostatic epithelium was normal, but some acini were composed of low inactive cells (Fig. 8). The pituitary acidophils, although smaller than normal, were larger than in the deficient rats.

Thymic involution resulting from protein inadequacy is a well known reaction. The response of the thymus to isoleucine deficiency followed a pattern similar to that observed in our earlier studies. Formation of giant cells, loss of normal architecture and depletion of thymocytes were observed (Fig. 9). Needle-shaped clefts, which are usually associated with cholesterol deposits, were present. Such clefts were seen also in tryptophan-deficient rats but not in rats deprived of threonine or histidine.

The thymuses of the pair-fed rats were smaller than normal, but the cortical and medullary zones were intact (Fig. 10). Giant macrophages were not present and no evidence of crystalline deposition was noted.

Degenerative changes in skeletal muscle were observed in the deficient animals. The areas of injury did not involve all fibers of the muscle which was examined (sternomastoideus). In all instances the damaged muscle fibers were losing, or had lost, their cross striations. Some damaged fibers were swollen and hyalinized (Fig. 11); in other fibers, hyalinization and fragmentation were most apparent (Fig. 12).

Discussion. In the light of the effect of isoleucine deficiency upon skeletal muscle, the recent study of Blahd, Bloom and Drell (16) is of interest. These investigators reported that one of the amino acid groups which appeared most frequently in the urine of male muscular dystrophy patients was isoleucine-leucine. Cole and Scott(17) noted that liver glycogen accumulation was high in tryptophan-deficient rats and that the glycogen content of the livers of pair-fed rats was low. Application of the glycogen staining method to liver sections of isoleucine-deficient rats showed that a large amount of glycogen was present (Fig. 13), while the hepatic cells of the pair-fed rats had little or none (Fig. 14). Quantitatively, the total liver glycogen content of the deficient group was 2.96 ± .15 mg/g and that of the pair-fed group was 0.57 ± .06 mg/g. The possible causes of

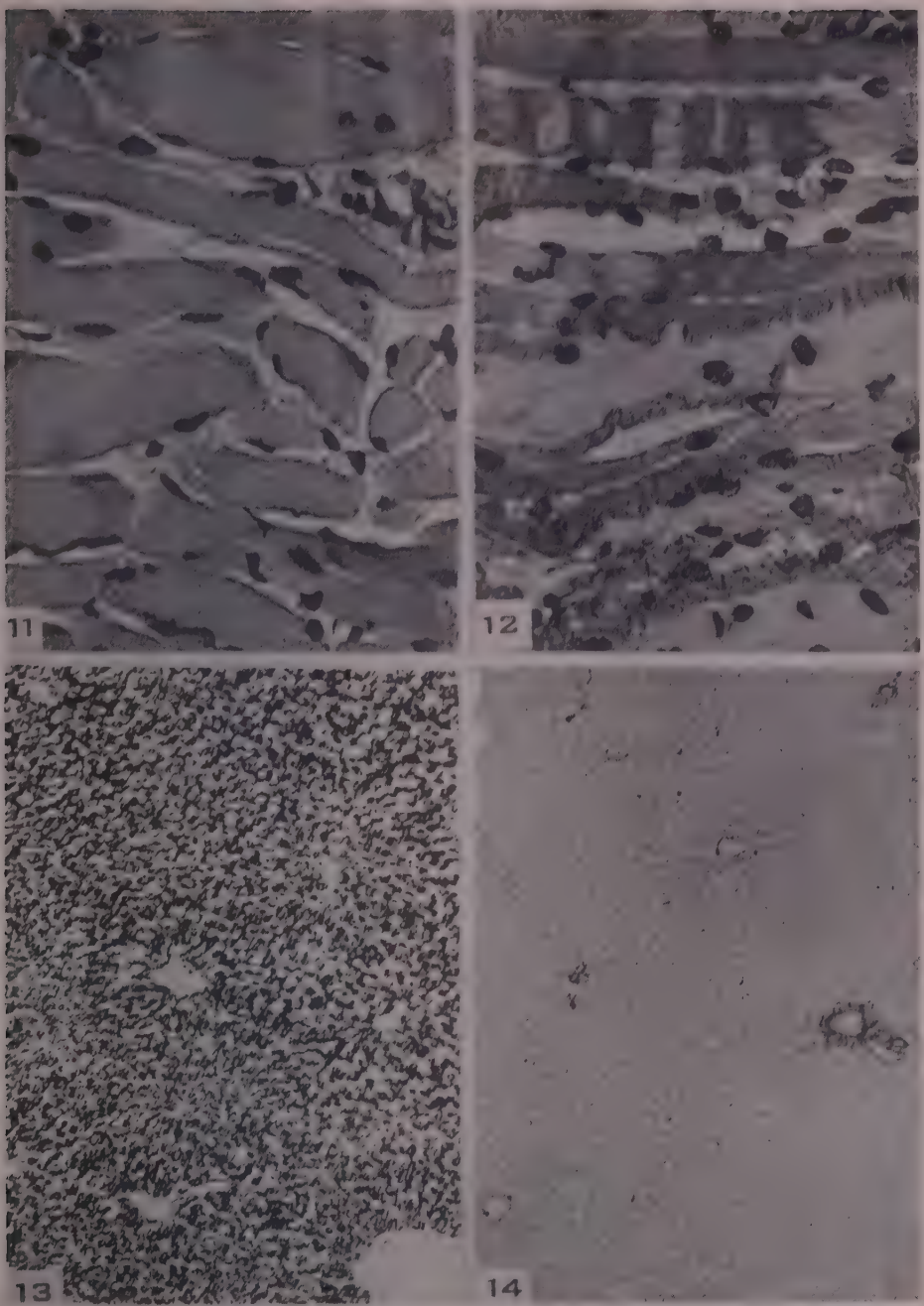

FIG. 11 and 12. Degenerating skeletal muscle fibers in isoleucine-deficient rats. ×430.

FIG. 13. Liver section of an isoleucine-deficient rat, stained by PAS method, illustrating presence of large amounts of intracellular glycogen. ×100.

FIG. 14. Liver section of a pair-fed control rat, stained by PAS method, showing absence of glycogen. ×100.

hepatic glycogen retention in amino acid deficient rats poses many problems for which there is no satisfactory explanation at this time. It is believed that the tissue alterations observed in the deficient rats were not the result of changes in water content.

The tissues of the 2 isoleucine-deficient rats which were placed on the recovery diet showed no abnormalities. Thus, the effects of the total lack of isoleucine were not permanent and were correctable by the addition of adequate dietary isoleucine.

Summary. The total lack of isoleucine in the diet of rats resulted in regression in the size of the pituitary acidophils, deletion of the pituitary gonadotrophic cells, atrophy of the testes and accessory sex glands, thymic involution, interference with somatic growth, liver glycogen accumulation and degenerative changes in skeletal muscle. Replacement of adequate isoleucine to the diet of isoleucine-deficient rats resulted in complete recovery.

The author is grateful to Dr. W. O. Read, Department of Physiology, for his assistance in making the total liver-glycogen determinations.

1. Womack, M., and Rose, W. C., *J. Biol. Chem.*, 1936, v116, 381.

2. Rose, W. C., and Rice, E. E., *Science*, 1939, v90, 186.

3. Whipple, G. H., and Robscheit-Robbins, F. S., *J. Exp. Med.*, 1940, v71, 569.

4. Madden, S. C., Anderson, F. W., Donovan, J. C., and Whipple, G. H., *ibid.*, 1945, v82, 77.

5. Rose, W. C., Haines, W. J., Johnson, J. E., and Warner, D. T., *J. Biol. Chem.*, 1943, v148, 457

6. Hegsted, D. M., McKibben, J. M., and Stare, F. J., *J. Clin. Invest.*, 1944, v23, 705.

7. Albanese, A. A., *J. Biol. Chem.*, 1945, v157, 613.

8. Rose, W. C., Oesterling, M. J., and Womack, M., *ibid.*, 1948, v178, 753.

9. Good, C. A., Kramer, H., and Somogyi, M., *ibid.*, 1933, v100, 485.

10. Halmi, N. S., *Endocrinology*, 1950, v47, 289.

11. Purves, H. D., and Griesbach, W. E., *ibid.*, 1951, v49, 244.

12. ———, *ibid.*, 1951, v49, 652.

13. Scott, E. B., and Schwartz, C., PROC. SOC. EXP. BIOL. AND MED., 1953, v84, 271.

14. Scott, E. B., *A. M. A. Arch. Path.*, 1954, v58, 129.

15. ———, *Am. J. Path.*, 1955, v31, 1111.

16. Blahd, W. H., Bloom, A., and Drell, W., PROC. SOC. EXP. BIOL. AND MED., 1955, v90, 704.

17. Cole, A. S., and Scott, P. P., *Brit. J. Nutrition*, 1954, v8, 125.

Received March 13, 1956. P.S.E.B.M., 1956, v92.

Vit. B_{12}: Correlation of Serum Concentrations and Pregnancy. (22411)

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Vit. B_{12} occupies a significant place in the nutrition of microorganisms, lower animals and man. It seems purposeful to seek clinical situations in which a deficiency of vit. B_{12} may be important, either as the sole factor or a contributing factor to the causation of the signs or symptoms which are observed. There is general agreement that a deficiency of vit. B_{12} exists in pernicious anemia (P.A.) and in this clear-cut clinical example of the deficiency state, the serum concentration of vit. B_{12} is a good index of the situation, being less than 100 $\mu\mu$g ml and approaching zero in frank untreated P.A. It is fully recognized that there is the possibility of a relatively normal serum concentration in the presence of deficiency in the total body stores and conversely the possibility of a lowered serum concentration in the absence of a measurable total body deficiency(1). Caution in interpreting serum vit. B_{12} concentrations is further enjoined on the basis of the accumulating evidence that when patients with P.A. in remission have had their vit. B_{12} serum concentrations assayed, the values may be below 100 $\mu\mu$g ml despite the fact that clinically the

patient is in remission(2). In view of the wide variation of vit. B_{12} serum concentrations from individual to individual when a presumably "normal" population is studied and, consequently, the probability that relatively large numbers of any "abnormal" group will have to be studied in order for a trend to achieve statistical significance, we have chosen to employ the vit. B_{12} serum concentration as the single criterion of potential deficiency in order to accelerate the "screening" of large numbers of people. It is hoped that the identification of populations found to be "abnormal" with respect to vit. B_{12} serum concentrations will contribute to the further investigation of these states and the establishment of whether the low serum vit. B_{12} is a direct measure of deficiency or of some other conditioning factor.

As a preliminary to studies of the type reported here, we have tried to ascertain something of the degree of variation between "normals" as well as the absolute values obtained. It was found and previously reported(3) that in a group of 528 individuals values below 200 $\mu\mu$g/ml or above 1000 $\mu\mu$g/ml were uncommon. The average was 560 $\mu\mu$g/ml with 95% confidence bands at 70 and 1,060 $\mu\mu$g/ml. By personal communication and subsequent publication(4) we learned of a tendency to lowered serum concentrations of vit. B_{12} having been observed during pregnancy in patients studied in Germany. Because of the possibility of therapeutic or prophylactic implications of such an observation, if this were related to the pregnant state independent of dietary or other variables, we were interested to attempt to corroborate and extend these data in the United States.

Methods and materials. Methods for the determination of vit. B_{12} where a titrimetric response is employed, such as the *L. leichmannii* method(5) will allow the direct assay of turbid and colored solutions. Previous studies have indicated that, whereas vit. B_{12} added to serum prior to "clarification" could not be recovered quantitatively, dilution without clarification did not affect recovery. Accordingly, we have employed the method previously described(4) of a microbiological assay with *L. leichmannii* and a dilution technic, which we believe represents "total serum vit. B_{12}." Patients from whom blood specimens were obtained were seen at one of two hospitals, namely, Abington Memorial Hospital (Hospital A) and Chestnut Hill Hospital (Hospital B). The method of handling specimens was essentially the same at each of these hospitals. Approximately 10 ml of blood were drawn from an antecubital vein and placed in a test tube which was allowed to stand in the ice box while clotting occurred and the serum separated. Later the same day the serum was transferred to a sterile 10 ml rubber-stoppered specimen vial and held in the deep freeze at –10°C until submission for assay. Specimens were coded at the time of freezing and decoded after the assays were reported. Beyond the routine dietary guidance given to pregnant patients, no attempt was made to regulate the diet or the vitamin supplementation of it in any of these patients. We recognized the vagaries of appetite of pregnant women as well as the sheer impossibility of rigidly controlling the intakes of so large a group of ambulatory individuals. Many of these patients received supplementary polyvitamin preparations by mouth, however, any patient who, in answer to questioning, indicated that she was receiving vit. B_{12} by injection, was excluded from the study. It was our feeling that vitamins by intramuscular injection represented a sufficient departure from the "average" of the middle class group of female patients who were followed in this study that inclusion of such patients would not be consonant with the objective of this study. None of these patients was toxemic at the time of blood collection. It was hoped that by sampling such a large group of women, the influence of variations in dietary intake and age would be minimized, and the duration of pregnancy became the only significant variable in terms of serum vit. B_{12}.

Results. The designation "days prior to delivery" represents the difference between the expected date of confinement (EDC), as calculated by the obstetrician, and the date of blood collection, with the exception of those

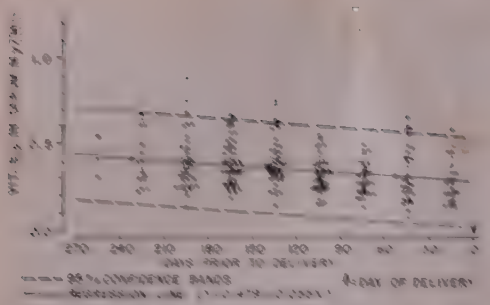

FIG. 1. Serum concentration of vitamin B_{12} as a function of duration of pregnancy in hospital A (318 females).

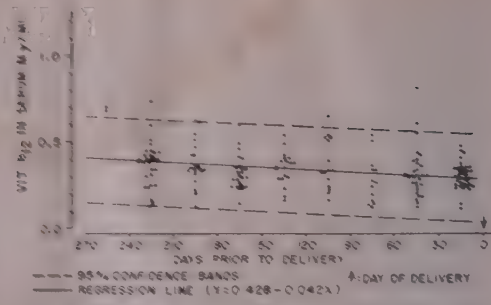

FIG. 2. Serum concentration of vitamin B_{12} as a function of duration of pregnancy in hospital B (184 females).

patients on whom blood was drawn at the time of delivery. Because of the considerable error involved in calculating the EDC, it seemed to us deceptively accurate to attempt to analyze the data according to the specific day of pregnancy. Accordingly, the individual values have been graphed according to 30-day periods and the statistical evaluation has been done according to 90-day periods.

Hospital A. Fig. 1 presents the individual serum concentrations and demonstrates the decline of the average value for serum vit. B_{12} in successive periods as the pregnancy approached term. Table I below presents the essential data from which the regression equation $Y = 0.479 - 0.055x$ was calculated where x is the period (1, 2 or 3). The slope, –0.055 mγ ml per period is highly significant statistically ($P < 0.001$).

Hospital B. Fig. 2 presents individual values and averages for 90-day periods and shows a decline of serum vit. B_{12} concentrations with duration of pregnancy similar to that seen in Hospital A. Table II below presents the essential data from which the regression equation $Y = 0.428 - 0.042x$ was calculated. The slope, –0.042 mγ ml per period is highly significant statistically ($P < 0.001$).

Discussion. The objective of this investigation was to correlate serum vit. B_{12} concentrations with duration of pregnancy. It seems evident from these results that a significantly lower serum vit. B_{12} level has been observed in the later stages of pregnancy. The lack of serum vit. B_{12} values within the "deficiency" range (under 100 μμg ml) is noteworthy. It is also of interest that the average serum B_{12} values for this entire group, even in the first trimester of pregnancy, was lower than the average established for "normal" males and females (3).

Many auxiliary determinations which might elucidate the mechanism involved were considered at the inception of the study but were deferred, because the extreme variation in serum vit. B_{12} concentration from individual to individual had been established and made

TABLE I. Summary of Data on 318 Pregnant Women in Hospital A.

	Period and days to delivery		
	1 (181–270)	2 (91–180)	3 (0–90)
No. of pregnant women	61	157	100
Avg B_{12} blood level (mγ ml)	.417	.377	.311
Expected or calculated level	.424	.369	.314
95% confidence limits for line of regression	.192–.583	.179–.629	.053–.575

TABLE II. Summary of Data on 184 Pregnant Women in Hospital B.

	Period and days to delivery		
	1 (181–270)	2 (91–180)	3 (0–90)
No. of pregnant women	51	57	76
Avg B_{12} blood level (mγ ml)	.384	.347	.300
Expected or calculated level	.386	.344	.302
95% confidence limits for line of regression	.133–.639	.093–.595	.050–.554

it likely that large numbers of patients would have to be studied. It seemed purposeful to confirm the existence of a lowered serum vit. B_{12} concentration in the pregnant state before more elaborate studies were undertaken.

It is well known that fluid retention and an expansion of plasma volume may occur in the later stages of pregnancy(6). It is possible that this may account for part or all of the decrease in the serum vit. B_{12} concentration which has been observed. It would seem necessary, however, to test the hypothesis that serum vit. B_{12} binding capacity might keep pace with hemodilution before concluding that hemodilution is the responsible factor. On the other hand, the increased requirement for many other vitamins of women during pregnancy is well established(7) and repeated pregnancies on a vit. B_{12} deficient diet have been the most effective way of inducing vit. B_{12} deficiency in animals(8).

The lowered serum vit. B_{12} concentrations that have been observed in this group of 502 pregnant women merits further study in order to ascertain the mechanisms involved.

Summary. 1. Serum vit. B_{12} estimations were made on sera obtained from 502 women after varying durations of pregnancy. 2. This work has confirmed previous suggestions that lower than normal serum vit. B_{12} concentrations are observed during pregnancy. It has also demonstrated a direct and statistically significant ($P<0.001$) correlation between serum level of vit. B_{12} and duration of pregnancy.

We are indebted to Dr. John Eiman, Abington Memorial Hospital, Abington, Pa. and Dr. S. Brandt Rose, Chestnut Hill Hospital, Philadelphia, Pa. for their generous cooperation in supplying serum specimens, to Mr. Joseph Ciminera for statistical analysis of the data and to Miss Gloria D. E. MacRae for the microbiological determinations.

1. Heinrich, H. C., *Klin. Wochenschr.*, 1954, v32, 867.

2. Reisner, Jr., Edward H., Weiner, L., Schittone, M. T., and Henck, E. A., *The New England J. Med.*, 1955, v253, 502.

3. Boger, W. P., Wright, L. D., Strickland, S. C., Gylfe, J. S., and Ciminera, J. L., PROC. SOC. EXP. BIOL. AND MED., 1955, v89, 375.

4. Heinrich, H. C., and Lahann, H., *Tagg Dtsch. Ges. Physiol. Chem. No.* 31, Homberg a.d. Saar, Oct. 1-4, 1953.

5. Skeggs, H. R., Nepple, H. M., Valentik, K. A., Huff, J. W., and Wright, L. D., *J. Biol. Chem.*, 1950, v184, 211.

6. Caton, W. L., Roby, C. C., Reid, D. E., and Gibson, J. G., *Am. J. Obstet. & Gyn.*, 1949, v57, 471.

7. National Research Council. Recommended Dietary Allowances, 1953.

8. Bosshardt, D. K., Paul, W. J., O'Doherty, K., Huff, H. W., and Barnes, R. H., *J. Nutrition*, 1949, v37, 21.

Received March 23, 1956. P.S.E.B.M., 1956, v92.

Effect of Local Anesthetics on Motility of Upper Gastrointestinal Tract.* (22412)

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Radiologists have reported that the intragastric administration of local anesthetic drugs results in relaxation of the pyloric sphincter. Bayer(1) gave peptic ulcer patients a 0.25% solution of larocaine orally. This was followed by relaxation of the previously closed pylorus and ready fluoroscopic visualization of the duodenum by contrast material passing from the stomach. Roka and Lajtha(2) used 100 cc of 1% solution of procaine in the same fashion and likewise demonstrated the opening of the pylorus by

* This work has been aided by Grant No. RG-4195, U. S. Public Health Service.

† John and Mary R. Markle Scholar in Medical Science.

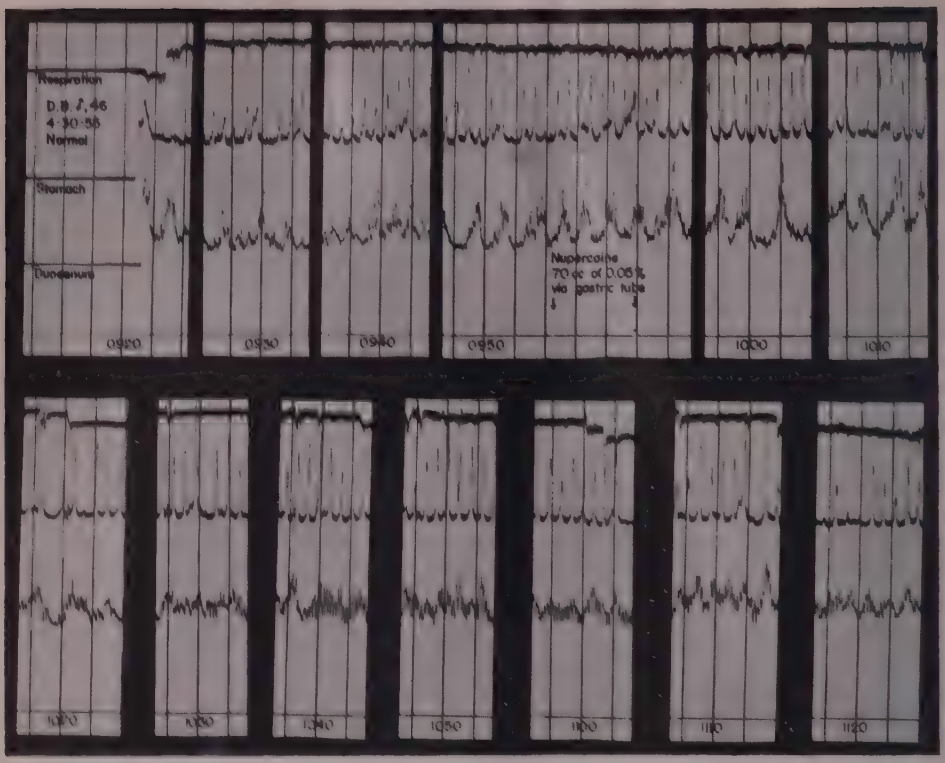

FIG. 1. Effect of locally administered nupercaine on gastric and duodenal motility.

fluoroscopic observations. Crohn, *et al.*(3) made kymographic measurements of gastrointestinal motility in both dogs and humans following administration of local anesthetics. In the dog, gastric motility was unchanged by metycaine or butyn, but small bowel motility was frequently inhibited by metycaine or procaine. In the human subject, they found reduction in gastric or intestinal motility in only a small proportion of their studies. Louckes and Quigley(4) administered procaine by stomach tube to dogs while measuring pyloric sphincter tone and motility. In lower doses (10-25 mg per kg) there was no effect; but larger concentrations often produced an increase in sphincter tone and a decrease in motility.

Method. Studies were performed on 9 human subjects, 7 of whom were entirely free of gastrointestinal disease, while 2 had chronic uncomplicated duodenal ulcer. Either the gastric or duodenal motility or both were recorded in each patient. A double lumen #14 F. rubber tube was passed under fluoroscopic control to the desired position(5); one lumen was used for inflating a balloon for recording motility and the other for the administration of the local anesthetic drug. Local anesthetics used were: 1) nupercaine (2-Butoxy-N-(2-diethylaminoethyl) cinchoninamide hydrochloride), 30 to 100 cc of a 0.05% solution in carmethose;‡ 2) pontocaine (tetracaine hydrochloride), 15 cc of a 0.5% aqueous solution; 3) elixir probutylin (procaine isobutyrate-Rorer), 10 cc totalling 1.000 mg.§ In 7 patients, the drug was instilled into the stomach and in 2 patients into the duodenum. Motility recordings were made by means of a multichannel recording oscillograph(6).

Results. 1. *Pontocaine*: One patient was given 75 mg (15 cc) of pontocaine intragas-

‡ Provided through the courtesy of Ciba Pharmaceutical Products, Inc., Summit, N. J.

§ Provided through the courtesy of William H. Rorer, Inc., Philadelphia, Pa.

trically. During the ensuing 100 minutes there was no change in either gastric or duodenal contractions.

2. *Elixir probutylin*: In one patient, 1000 mg (10 cc) of elixir probutylin instilled into the stomach was followed by no change in the gastric motor activity for over 2 hours. In a second patient, the same dose infused into the stomach resulted in a decreased frequency of gastric contractions with no change in amplitude and no effect on duodenal motility.

3. *Nupercaine*: Thirty to 100 cc of 0.05% nupercaine were given to 6 patients. The results were as follows: a) 30 cc instilled into the stomach of one patient with duodenal ulcer produced no change in gastric activity in 90 minutes; b) 50 cc of nupercaine introduced into the duodenum of the second patient with duodenal ulcer was followed by no change in either gastric or duodenal motility during a 50-minute period; c) 70 cc of nupercaine infused into the stomach had no effect on gastric motility but duodenal motility changed from type II to alternating type I and II (Fig. 1); d) 75 cc of nupercaine instilled into the duodenum resulted in no change in duodenal activity for 90 minutes; e) 75 cc of nupercaine dripped into the stomach was followed by no change in gastric contractions until 50 minutes, when the amplitude became moderately reduced; and f) 100 cc of nupercaine instilled into the stomach was followed by no change in gastric motor activity for over 2 hours.

Discussion. Direct administration of local topical anesthetic agents into the stomach and duodenum of 9 human subjects had no discernible effect on the gastric and/or duodenal motility in 6 of them. In 3 patients there were minor and seemingly inconstant changes in gastric or duodenal activity; in each case the trend was toward a decrease. Tonus and motility of the pyloric sphincter were not measured.

As previously reported(7) topical anesthetic drugs prevent or ameliorate acid-induced peptic ulcer pain. This relief is seemingly not related to a change in motor activity. The pyloric sphincter relaxation reported on the basis of fluoroscopic observation following oral administration of local anesthetics cannot be explained on the basis of the present study since there was no significant change in recorded gastroduodenal motility.

Summary and conclusions. Local topical anesthetics, nupercaine, elixir probutylin, and pontocaine, were administered into the stomach or duodenum of 9 human subjects. Gastric and/or duodenal motility was recorded oscillographically and found to be unchanged in 6 patients; minor and seemingly inconstant changes occurred in 3 of the 9 subjects.

1. Bayer, L., *Zentralbl. f. Inn. Med.*, 1934, v55, 577.

2. Roka, G., and Lajtha, L. G., *Brit. Med. J.*, 1950, v2, 1174.

3. Crohn, N., Olson, W. H., and Necheles, H., *Surg., Gynec. & Obst.*, 1944, v79, 41.

4. Louckes, H. S., and Quigley, J. P., *Fed. Proc.*, 1955, v14, 95.

5. Woodward, E. R., and Schapiro, H., PROC. SOC. EXP. BIOL. AND MED., 1954, v86, 504.

6. Bachrach, W. H., Brody, M., and Drury, D. R., *Gastroenterology*, 1951, v18, 609.

7. Woodward, E. R., and Schapiro, H., *J. Appl. Physiol.*, 1956, v8, 357.

Received March 26, 1956. P.S.E.B.M., 1956, v92.

Potentiation of Urethan Anesthesia by Epinephrine. (22413)

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During a study of the action of drugs on carotid blood pressure in mice under urethan anesthesia(1), it was observed that they remained under deep anesthesia for a long time following a dose of epinephrine. Further experiments were thereupon conducted to investigate the effect of epinephrine on urethan anesthesia. This report presents the results of such experiments and shows that epinephrine potentiates urethan anesthesia, and that urethan reduces lethal toxicity of epinephrine.

Method and materials. Experiments were conducted in 3 strains of mice (CAF_1, C3H and C57 Black), weighing 18 to 24 g, and of both sexes. Urethan (10% solution) and, usually after an interval of one hour, epinephrine (0.05 and 0.1% solution), were injected in various doses either subcutaneously or intraperitoneally. Phenobarbital sodium and pentobarbital sodium were given in various doses in some experiments. Duration of anesthesia was measured as the time elapsed between the loss of righting reflex and its return(2-4). This interval should really be called "side position time" (SPT) rather than "anesthetic or sleeping time"; but since the latter term implies the presence of a state which is difficult to measure in mice(2), SPT has been used in these experiments to signify "anesthetic time."

Results. Table I summarizes the results of experiments on epinephrine toxicity in the 3 strains of mice. A dose of 5 μg/g killed 24% of CAF_1 mice, 57% of C3H mice, and 100% of C57 Black mice. From the overall picture at all dose levels, the lethal toxicity appeared to be lowest in the CAF_1 mice and greatest in the C57 Black strain.

Toxic manifestations following injection of epinephrine, especially of high doses, were: apprehensiveness, erection of hair, bulged eyeballs, gradually increasing prostration, frothing (sometimes hemorrhagic) through the nostrils, increased respiration, restlessness, and slight excitatory movements before death.

To study the effect of combination treatment, C57 Black mice were given a subcutaneous dose of urethan followed, after one hour, by an intraperitoneal dose of epinephrine. Results of experiments with various dose combinations show that duration of anesthesia in the survivors increased with the dose of either drug (Table II).

Moreover, urethan protected the mice against the lethal toxicity of epinephrine and prolonged their survival time. Under urethan anesthesia, the toxic manifestations of epinephrine were present, but they were less marked. With epinephrine alone, all but one of the mice died within a short time; the interval from injection to death was 2 ± 1 hr., 1.5 ± 0.5 hr., and 1 ± 0.1 hr. for the 3 dose levels used (2.5, 5 and 10 μg/g, respectively). None of these mice, of course, were anesthetized. At all doses of epinephrine, combination with urethan reduced the number of deaths. Moreover, the mice that died, following injection of urethan and the 2 higher doses of epinephrine, survived longer than those that got epinephrine alone. At the highest dose of epinephrine, the survival was prolonged with epinephrine up to a maximum of 5 hr beyond the epinephrine controls.

In other experiments, also with C57 Black mice, epinephrine was given in 3 dose levels (20, 10 or 1 μg/g) alone, or in combination with various dose levels of urethan. At the 2 higher levels of epinephrine, all the mice died. With 1 μg/g of epinephrine no deaths occurred; the anesthetic time was again prolonged by combination with the higher doses of urethan (1.4 and 1.6 mg/g).

The experiment was repeated with CAF_1 mice, in which epinephrine was less toxic.

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TABLE I. Lethal Toxicity* of Epinephrine in 3 Mouse Strains.

Strain	Route	Ratio of: No. dead/No. treated					
		1 μg/g	2.5 μg/g	5 μg/g	10 μg/g	15 μg/g	20 μg/g
CAF_1	Subcut.	—	0/ 6	3/12	9/12	6/ 6	6/ 6
"	Intraper.	—	—	5/22	12/12	29/38	21/22
C3H	Subcut.	0/5	1/ 5	19/34	7/ 8	8/ 8	8/ 8
C57 Black	Subcut.	0/6	2/ 6	6/ 6	6/ 6	—	—
" "	Intraper.	0/6	8/12	12/12	12/12	—	—

* Toxicity was studied usually on 5 or 6 mice at each dose level; larger numbers at certain doses signify pooled data from several experiments.

Epinephrine (2.5, 5, 10, 15 or 20 μg/g) was injected intraperitoneally one hour after a subcutaneous dose of urethan (1.5 mg/g).

In this experiment the data on anesthesia and mortality were recorded at 0.5-1 hour intervals up to 6 hours and again after 24 hours. Table III gives the detailed data on combination of a fixed dose of urethan with various doses of epinephrine. It shows that preadministration of urethan completely protected the mice against the lethal toxicity of low doses of epinephrine (*cf.* Table I); as the dose level of the latter increased to 10 μg/g or more, the degree of protection decreased. In Fig. 1, curve A shows the relationship between the dose of epinephrine and the percentage reduction of its lethal toxicity by urethan.

Curve B in Fig. 1 shows the potentiating effect of epinephrine on duration of anesthesia of urethan; the potentiation increased with the dose, as also can be seen from Table III. This lowering of epinephrine lethal toxicity by urethan and the prolongation of urethan anesthesia by epinephrine, in CAF_1 mice, is in agreement with the results obtained in C57 Black mice. With these drugs in combination, at the same dose levels, percent survival was higher in CAF_1 mice, but prolongation of urethan anesthesia was less than in the C57 Black strain.

From these curves it is seen that this combination gave an optimum effect at a dose level between 5 and 10 μg/g of epinephrine. At lower doses, prolongation of anesthesia was less marked and the proportion of mice having a long period of anesthesia was less, while higher doses gave increasing mortality.

In the combination treatment, reversal of route of administration, *i.e.* urethan given intraperitoneally and epinephrine subcutaneously, produced similar effects, but they were less marked. Similar experiments were carried out in all 3 strains of mice, with similar results. The most complete set of experiments was carried out in C3H Black mice, in which the largest number of dose variations was employed (urethan at 1.2, 1.4 or 1.6 mg/g; epinephrine at 5, 10, 15 or 20 μg/g). The results obtained in all of these experiments were entirely analogous to those obtained when the urethan was given subcutaneously and the epinephrine given intraperitoneally, except that the reduction in epinephrine mortality and the prolongation of urethan anesthesia were less pronounced.

Phenobarbital sodium (100 μg/g) or pentobarbital sodium (45, 60 or 75 μg/g), injected subcutaneously half an hour before an intraperitoneal injection of epinephrine (5 μg/g) in C3H and C57 Black mice, did not reduce the lethal toxicity of the latter; but the survival period was slightly prolonged. With this combined treatment, however, the right-

TABLE II. Duration of Anesthesia in Surviving Mice* (C57 Black).

Epinephrine (μg/g)–intraper.	Hr			
10	—	>6	>24	>24
	(0)	(2)	(1)	(1)
5	—	>6	> 6	>24
	(0)	(4)	(4)	(3)
2.5	0	1.5 ± .5	4.5 ± 1.0	6.0 ± .2
	(1)	(6)	(5)	(6)
0	—	0	0	1.5 ± .5
		(6)	(6)	(6)
	0	1.2	1.4	1.6
	Urethan (mg/g)–subcut.			

* Number in parenthesis indicates No. of survivors in each group of 12 mice.

TABLE III. Anesthetic Time of Urethan (1.5 mg/g), Alone or Combined with Epinephrine, in CAF_1 Mice.

Dose of epinephrine, μg/g	No. of mice remaining under anesthesia for						
	0 hr	to .5 hr	.5-1 hr	1-2 hr	2-4 hr	4-6 hr	6 hr
0	10				2		
2.5	6	3		2	1		
5		1			1	6	4
10	1	(1)		(1)	(1)		6 (2)
15		(6)		(1)		1	4
20		(6)	(1)	(2)			2 (2)

At each dose level, 12 mice were employed.
Numbers within parenthesis show the mice that died under anesthesia within the respective periods.

ing reflex became negative following the injection of epinephrine and remained unchanged till death. Epinephrine (2.5 μg/g) in these mice increased the anesthetic time of pentobarbital; the increase was greater, the higher the dose of pentobarbital.

Anesthesia produced in C3H mice with urethan (1.5 mg/g-subcutaneous) was markedly prolonged also by nor-epinephrine (20 μg/g-intraperitoneal), and the lethal toxicity of nor-epinephrine was somewhat reduced by urethan. Cortisone (50 μg/g) did not potentiate the action of urethan (1.2 mg/g) in C57 Black mice.

Discussion. Increasing interest has been shown recently in the potentiating effect of various sedative and non-sedative agents on anesthesia induced by barbiturates(2-6,8) and by other anesthetics(6,13,9). While the effect of the sedative agents can be explained on the basis of their synergistic depressive action on the central nervous system, the mechanism of action of the non-sedative agents is still obscure. The effect of several anesthetics has been potentiated by non-sedative agents of diverse nature, such as epinephrine(9,11), nor-epinephrine(9), histamine(6), glucose(8,10), intermediary metabolites(8,9,10) and various other organic and inorganic substances(2,5,8). Contradictory findings are, however, not lacking to show that epinephrine shortens the sleeping time (7). A substance found to be an effective potentiating agent with one anesthetic may fail with another(5,10). Sodium chloride potentiated barbiturate anesthesia, but failed in case of urethan(5).

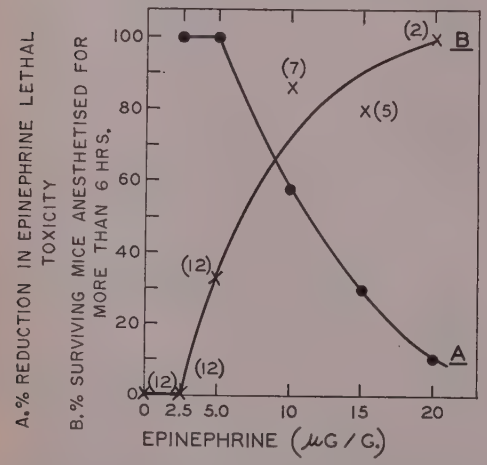

FIG. 1. Reduction of epinephrine lethal toxicity by urethan (1.5 mg/g) (curve A) and potentiation of urethan anesthesia by epinephrine (curve B) in CAF_1 mice. Numbers in parenthesis indicate No. of survivors at the end of 24 hr, in a group of 12 mice each.

In our experiments with mice, potentiation of pentobarbital anesthesia by epinephrine was slight; whereas, anesthetic time of urethan was markedly prolonged by epinephrine and nor-epinephrine. Variation in lethal toxicity of epinephrine in different strains of mice was noticed as in the case of the response to hexobarbital(12). In a given strain of mice the more toxic the dose of epinephrine the more prolonged was the anesthetic time of urethan in combination with epinephrine. For a particular dose of epinephrine, the more susceptible a species to epinephrine toxicity the more marked was the potentiating effect of epinephrine on urethan anesthesia.

Attempts have been made from time to time to explain the mechanism of the poten-

tiation of anesthesia by these non-sedative agents. Experiments(7-9) to investigate whether interference with carbohydrate metabolism, by various agents including epinephrine, is the cause of potentiation of anesthesia have not been conclusive. In an exploratory experiment, we injected insulin (0.4 unit/mouse) intraperitoneally in C3H mice under urethan with or without epinephrine; it did not counteract the effect of epinephrine on urethan anesthesia. This experiment did not indicate that the action of epinephrine on carbohydrate metabolism was responsible for prolongation of urethan anesthesia. Permeability of the brain to barbiturates was found (8) to be increased by lactate, pyruvate and glutamate but not by other substances examined. It has also been shown that potentiating effect of epinephrine is not linked with its action on adrenal cortex(11). On the other hand, barbital anesthesia(18) in mice was found to be prolonged by adrenalectomy; pretreatment with cortisone returned the anesthetic time to normal level, but hydrocortisone decreased it significantly. These effects on anesthesia were found to be correlated with barbital concentration in brain.

The various actions of epinephrine on the central nervous system are difficult to reconcile with each other. The conventional therapeutic dose has little stimulating effect on the central nervous system, but large doses or small doses injected into the carotid artery in animals manifest a stimulating effect(14). Epinephrine was found to potentiate the convulsant effect of electroshock(15). On the other hand, it has some analgesic effect(14) and can cause anesthesia in dogs after intracisternal injection of quite large doses(16,17). In our experience, a few mice were found to lose the righting reflex and appeared to be under anesthesia after a large intraperitoneal dose of epinephrine alone. On the basis of these diverse findings, it is difficult to ascribe the potentiating effect of epinephrine to its action on the central nervous system, and the mechanism of its action on anesthesia remains to be resolved.

Summary. Three strains of mice (CAF_1, C3H and C57 Black) were treated with various dose combinations of urethan and epinephrine. Duration of anesthesia, lethal toxicity and survival time were observed, with the following findings: 1. Epinephrine lethal toxicity was greatest in C57 Black mice and lowest in the CAF_1 strain. 2. In all strains, urethan anesthetic time was greatly prolonged; this effect increased with dose of epinephrine. 3. Urethan reduced lethal toxicity of epinephrine; with increase of dose of epinephrine, reduction of toxicity was less. 4. Urethan anesthetic time was also potentiated by nor-epinephrine; its lethal toxicity was somewhat reduced by urethan. 5. Anesthetic time of phenobarbital sodium and of pentobarbital sodium was slightly potentiated by epinephrine. These agents did not reduce the lethal toxicity of epinephrine.

1. Pradhan, S. N., Achinstein, B., and Shear, M. J., *J. Pharm. Exp. Therap.,* 1955, v113, 44.

2. Kahn, Jr., J. B., *ibid.,* 1953, v109, 292.

3. Cook, L., Toner, J. J., and Fellows, E. J., *ibid.,* 1954, v111, 131.

4. Goldin, A., Dennis, D., Venditti, J. M., and Humphreys, S. R., *Science,* 1955, v121, 364.

5. Richards, R. K., Bertcher, E. J., and Taylor, J. D., *Arch. int. Pharmacodyn.,* 1952, v89, 463.

6. Ambrus, J. L., Ambrus, C. M., Leonard, C. A., Moser, C. E., and Harrison, J. W. E., *J. Am. Pharmaceut. Assn.,* 1952, v41, 606.

7. Westfall, B. A., *J. Pharm. Exp. Therap.,* 1946, v87, 33.

8. Lamson, P. D., Greig, M. E., and Hobdy, C. J., *ibid.,* 1951, v103, 460.

9. Lamson, P. D., Greig, M. E., and Williams, L., *ibid.,* 1952, v106, 219.

10. Lamson, P. D., Greig, M. E., and Robbins, B. H., *Science,* 1949, v110, 690.

11. Fenu, G., *Minerva Otorinolaringologica,* 1954, v4, 91.

12. Jay, G., PROC. SOC. EXP. BIOL. AND MED., 1955, v90, 379.

13. Heinrich, M. A. Jr., *Fed. Proc.,* 1951, v10, 307.

14. Goodman, L. S., and Gilman, A., *The Pharmacological Basis of Therapeutics,* The Macmillan Co., New York, 1955, 490.

15. Minz, B., and Domino, E. F., *J. Pharm. Exp. Therap.,* 1953, v107, 204.

16. Leimdorfer, A., and Metzner, W. R. T., *Am. J. Physiol.,* 1947, v150, 588.

17. ———, *ibid.,* 1949, v157, 116.

18. Komiya, A., and Shibata, K., *J. Pharm. Exp. Therap.,* 1956, v116, 98.

Received March 26, 1956. P.S.E.B.M., 1956, v92.

Formation in a Mycobacterium of an Adaptive Enzyme for Oxidation of Phloroglucinol. (22414)

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The rate of adaptive enzyme formation in micro-organisms is influenced by a number of conditions. Provided that permeability is not a limiting factor, the metabolic and nutritional state of the cell may be the most important in determining the rate. Washed suspensions of a species of mycobacterium form enzymes for the oxidation of benzoic acid and inositol(1). Under the same conditions the formation of the inositol enzyme takes a much longer time. It appears that it is more difficult for the cell to make this enzyme because the age of the culture, the presence of assimilable nitrogen, and certain drugs affect inositol enzyme formation much more than they do benzoate enzyme formation. It has now been shown that this organism makes an enzyme which oxidizes phloroglucinol and which is different from the other two in that it is formed more slowly and the factors which affect the inositol enzyme formation, with exception of the drugs, have little effect on the formation of the phloroglucinol enzyme. Thus, under conditions which are apparently optimal for the formation of 2 enzyme systems, the formation of the third is unaltered from the original basic rate.

Methods. Mycobacterium BCG ATCC No. 8420 was grown for 3 to 7 days on 20 ml of Long's synthetic medium(2). The cell mass was broken up, washed twice with water by centrifugation in Hopkin's tubes, and then taken up in 0.05 M Na-K phosphate buffer pH 7.8 so that 0.1 ml of packed cells was suspended in 1 ml of buffer. Each Warburg vessel, which had a fluid volume of 2 ml, contained 0.5 ml of this suspension.

Results. Two criteria were used to show that the delay in the oxidation of phloroglucinol was the result of slow enzyme formation rather than slow penetration into the cell. Low concentrations of antibiotics inhibit enzyme formation in this organism(1) but have no effect on the oxidation once the enzyme is formed. In this case, 0.05 γ/ml of aureomycin completely prevented the oxidation of phloroglucinol when added with it but was without effect on the rate when the drug was added after the oxidation was proceeding. Then, pre-incubation of the cells for 90 minutes with 50 γ of phloroglucinol decreased the latent period when 0.5 mg of phloroglucinol was added. On the other hand, pre-incubation with benzoate or inositol had no effect. Further, cells grown in benzoate or inositol did not oxidize phloroglucinol more rapidly. Phloroglucinol reacts with the medium to produce a colored complex and this may explain why the enzyme is not formed when grown in the presence of phloroglucinol.

Phloroglucinol is toxic. This is seen from the following figures for the oxygen uptake in a 2-hour period with addition of 0.1, 0.5, 1.0, and 2.0 mg of phloroglucinol per vessel. In cmm of oxygen these were, respectively, 54, 87, 72 and 53. This toxicity makes it difficult to assess the effect of various factors on enzyme formation since different conditions may alter the resistance of the cells to the toxic effect. In general, it can be stated that the age of the culture from which the cells were taken had no consistent influence on the length of the latent period. This is in contrast to the effect of age on the rate of formation of the benzoate and inositol enzymes. Pre-incubation of the cells with $(NH_4)_2SO_4$, which has a marked effect on the formation of the inositol enzyme, had little or no effect on the formation of the phloroglucinol enzyme. Pre-incubation with trehalose, pyruvate, fatty acids, asparagin, catechol, and pyrogallol was also without effect.

Neither resorcinol nor phenol was oxidized even if the cells were pre-incubated with phloroglucinol. Cells grown in 50 γ/ml of resorcinol or phenol were unable to oxidize them nor were these cells able to oxidize phloroglucinol more rapidly. Normal cells,

TABLE I. Oxidation of 0.4 mg Each of Phloroglucinol and Orcinol. Pre-incubation period was 90 min., pH 7.8, 37°. Figures are mm^3 oxygen uptake. Autorespiration has been subtracted.

Compound	Pre-incubated with	Min. 30	60	90	120	150	180
Phloroglucinol	—	−4	10	23	53	77	98
"	50 γ phloroglucinol	10	24	38	72	99	129
"	50 γ orcinol	−4	12	25	56	81	108
Orcinol	—	−9	−9	−6	−5	15	34
"	50 γ phloroglucinol	−1	14	39	72	108	143

however, oxidized orcinol after a long latent period which could be shortened by pre-incubation with phloroglucinol. This is shown in Table I in comparison with the oxidation of phloroglucinol. Pre-incubation with orcinol had only a slight effect on the latent period for phloroglucinol. For the formation of the enzyme, therefore, 3 hydroxy groups meta to one another on the ring are necessary. If one was substituted by a methyl group as in orcinol, the enzyme was formed much more slowly, but the enzyme once formed was able to oxidize orcinol fairly rapidly. The substitution of a carboxyl group as in 3, 5, dihydroxybenzoic acid caused complete loss of activity. It did not cause formation of the enzyme, was not oxidized by it, nor did it inhibit formation or oxidation. This was also true of phloridzin which is a glucoside of phloroglucinol. Enzyme formation occurred at the normal rate in the absence of added phosphate.

The oxygen uptake varied between 4 and 6 atoms per molecule of phloroglucinol and 2 molecules of carbon dioxide per molecule were produced. The ring was therefore split and some assimilation of the end products, if it occurred, could account for the variability of the oxygen uptake. It is probable that more than one enzyme is involved in the overall reaction.

Summary. The rate of formation of the enzyme which oxidizes phloroglucinol is not affected by factors which alter the rate of formation of the enzymes which oxidize benzoic acid and inositol. Some of the characteristics of the phloroglucinol oxidation are described.

1. Ottey, L., and Bernheim, F., *Enzymologia,* in press.

2. Long, E. R., *Amer. Rev. Tuberc.,* 1926, v13, 393.

Received March 26, 1955. P.S.E.B.M., 1956, v92.

Effect of Adrenocorticotropin on Uptake of S^{35}-Labelled Cysteine by Rat Adrenal Glands.* (22415)

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Reports of the effects of the adrenocorticotropic hormone (ACTH) upon sulfur distribution in the animal body have been largely confined to extra-adrenal tissues and fluids. Kinsell *et al.*(1) demonstrated a 50% rise in urinary cystine and cysteine after the administration of the hormone. Hess *et al.* (2) found a marked fall in the concentration of reduced glutathione in blood but no change in total glutathione levels after administration of ACTH to human patients. Ingbar *et al.* (3) found that the hormone produced no changes in the blood glutathione of rats although it did cause a reduced non-protein

* Supported by grant from the Milton Fund of Harvard University and by Contract No. AT (30-1)-609 from U. S. Atomic Energy Commission.

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sulfhydryl level in the kidney and an increased level in the liver. No changes in the amount of protein sulfhydryl were observed. Goldzieher *et al.*(4) observed a 50% rise in rat adrenal non-protein sulfhydryl concentration 3 hours after injection of 0.1 mg of Armour ACTH, but a reduced sulfhydryl level after larger doses or longer treatment. Liver and diaphragm exhibited no such changes at the lower dose, but non-protein sulfhydryl content was reduced with the larger amounts of hormone. In distribution studies by Lee and Williams(5) ACTH was demonstrated to increase the incorporation of S^{35}-labelled DL-cystine in a number of tissues of the rat. No studies were made with the adrenal itself. The present investigation was undertaken to study the *in vitro* effect of this hormone upon uptake and distribution of cysteine in the rat adrenal gland.

Materials and methods. The animals used were 180 to 200 g female Sprague-Dawley rats. Adrenal glands were removed, halved and incubated for 20 minutes at 37°C in an artificial medium consisting of 0.025 M NaCl, 0.05 M KH_2PO_4, 0.015 M K_2HPO_4, and 0.01 M glucose. The pH was 7.25. An oxygen atmosphere was present within the flasks. After incubation the adrenals were filtered onto glass wool and thoroughly rinsed with normal sodium chloride solution. About one-third of the tissue was set aside for oxidation of the total sulfur content to sulfate. The remainder was fractionated into lipid, protein and water-soluble fractions according to the methods of Folch(6). The sulfur of these fractions was also oxidized to sulfate. In experiments with labelled cysteine carrier sulfate was added and the sulfate was precipitated with benzidine by conventional methods and counted. In early experiments, oxidation was carried out in open tubes by successive treatment with hydrogen peroxide-glacial acetic acid, aqua regia and finally perchloric and nitric acids. Since considerable variation and poor recoveries were obtained by this procedure, oxidation with aqua regia in a sealed Carius tube as described by Lees (7) was adopted and proved more satisfactory. The samples of whole adrenal gland and fractions were dried and weighed in 125 mm x 14 mm combustion tubes. Small test tubes (30 mm x 6 mm) containing one-half ml of freshly prepared aqua regia (three parts concentrated nitric acid to one part of concentrated hydrochloric acid) were introduced into the large tubes. This amount was sufficient to oxidize twenty mg of organic material. Combustion tubes were drawn out, sealed and inverted to permit contact of the sample and oxidizing agent. The tubes were wrapped in asbestos paper and placed in iron bombs (capped one inch pipes) in an oven at 250°C for 8 hours. The tubes were opened in the hood and the contents carefully washed out and evaporated to dryness for precipitation. When benzidine sulfate was to be determined, rather than counted, the colorimetric method of Letonoff and Reinhold was used(8). L-cysteine hydrochloride was a sulfur-labelled preparation by Abbott Laboratories. The ACTH used was an Astwood preparation, assaying 80 International Units per milligram.

Results. Values for total and fractional solids and total and fractional sulfur contents of the rat adrenal were determined initially so that calculations of uptakes could be made on weight basis. The sulfur content of the rat adrenal has been reported to be 1.5% of the dry weight(9). This would far exceed that of any other tissue and repetition of this determination seemed desirable. Accordingly sulfur analyses were performed upon the normal adrenal by the closed Carius method. A few independent checks by oxidation of samples in the Parr bomb under thirty atmospheres of oxygen agreed very well, and these data were pooled with the Carius data. The adrenals of 3 rats were used for each total sulfur determination; those of 5 rats for the fractionation studies. The results are seen in Tables I and II. The data show much less sulfur than had been previously reported(9).

Experiments were then carried out to determine whether or not the adrenal gland took up cysteine *in vitro*, how it was distributed among the protein, lipid and water-soluble fractions and the effect of added ACTH on the uptake and distribution. In preliminary

TABLE I. Solid Content of Rat Adrenal Gland.

Component of gland	No. of determinations	Avg % of wet wt	Stand. error of mean	% of total solid
Whole	20	29.0	.51	
Protein	16	14.8	.28	51.0
Lipid	15	12.4	.34	42.8
Water-soluble	15	2.8	.31	9.5
			Recovery	103.3

TABLE II. Sulfur Content of Rat Adrenal Gland.

Component of gland	No. of determinations	Avg % of dry wt	Stand. error of mean	% of adrenal sulfur
Whole	13	.633	.032	
Protein	8	.467	.026	73.9
Lipid	9	.025	.006	4.0
Water-soluble	9	.177	.024	28.0
			Recovery	105.9

experiments using both inactive and radioactive cysteine, the disappearance of the substrate from the medium was too small to measure with accuracy. Therefore, the actual amount of radioactive cysteine taken up was measured by oxidizing the glands themselves.

The adrenal glands of 6 rats (290 mg) were incubated in each of 4 flasks for 20 minutes in 3 ml of the buffered medium. S^{35}-labelled cysteine was present in each flask at a concentration of 10 mg%. Duplicate incubations were run for each value reported. Two of the flasks contained ACTH at a concentration of 5 International Units per 100 mg of tissue. The total uptake of radioactivity averaged 4.62% in the flasks with cysteine alone, 5.20% with cysteine and the hormone. The distribution of radioactivity found is shown in Table III.

The initial activity of the medium was 3.2 x 10^5 counts per minute per ml, or 1.02 x 10^6 counts per minute per mg S as cysteine. Computations of the radioactivity which could be present in the glands if all the water (71% of the adrenal weight) was in equilibrium with the medium gives a value of 66,000 counts per minute. The actual values found were 44,300 and 50,000 counts per minute for the cysteine and cysteine plus ACTH samples respectively.

Discussion. On the basis of these experiments it appears that ACTH causes a marked change in the uptake of labelled cysteine from the water-soluble fraction of the gland to the lipid and protein fractions. Total uptake was also increased slightly, in agreement with the S^{35} cystine data on other tissues(5).

The increase in total uptake might be expected from adrenal stimulation under the influence of ACTH, but the significance of the rise in radioactivity of lipid sulfur and indeed of the very striking amounts of activity in the lipid sulfur as compared to the protein is interesting. It might indicate a very rapid turnover of some lipid, possibly proteolipid. However, the possibility of cysteine or some other sulfhydryl compound reacting with steroids or other lipid components must not be overlooked. The formation of thiazolidines has been reported by the interaction of cysteine with many steroids under very mild conditions in the test tube(10). Whether this might have a physiological importance is impossible to state at this time.

Summary. Values for total sulfur content of the rat adrenal gland and its protein, lipid and water-soluble moieties have been deter-

TABLE III. Effect of ACTH Preparation upon Uptake of Labelled Cysteine by Rat Adrenals.

Component of adrenal	Cysteine alone (I) Counts/min. /mg dry adrenal	Cysteine alone (I) % of total	Cysteine alone (I) Counts/min. /mg S of fraction $\times 10^4$	Cysteine plus ACTH (II) Counts/min. /mg of adrenal	Cysteine plus ACTH (II) % of total	Cysteine plus ACTH (II) Counts/min. /mg S of fraction $\times 10^4$	Difference II – I %
Whole	527 ± 85.0*		8.33	595 ± 45.0*		9.40	+13.0
Protein	118 ± 4.1	22.4 ± .8*	2.52	194 ± 24.0	32.6 ± 4.0*	4.16	+64.5
Lipid	36.2 ± 8.5	6.9 ± 2.4	14.5	48.9 ± 2.7	8.2 ± .5	19.5	+35.1
Water-soluble	267 ± 26.2	50.7 ± 5.0	15.6	185 ± 25.7	31.1 ± 4.3	10.4	−30.6
Recovery		80.0			71.9		

* ± Stand. error of mean.

mined. The total sulfur as determined (0.63% of the dry weight) is much lower than previously reported values. Adrenal glands were incubated briefly with radioactive cysteine and the tissue was then analyzed for total uptake of the isotope and its distribution within the fractions. The effect of added adrenocorticotropin upon this uptake and distribution was observed. The hormone preparation causes a marked reduction in uptake by the soluble fraction, and a rise in the uptake by the lipid and protein fractions. The concentration of the isotope in the lipid sulfur is very striking.

We are grateful to Dr. E. B. Astwood for generous supplies of his adrenocorticotropic hormone preparation.

1. Kinsell, L. W., Li, C. H., Margen, S., Michaels, G. D., and Hedges, R. N., *Proc. First Clinical ACTH Conference,* Blakiston Co., 1950, 70.

2. Hess, W. C., Kyle, L. H., and Doolan, P. D., PROC. SOC. EXP. BIOL. AND MED., 1951, v76, 418.

3. Ingbar, S. H., Otto, J. F., and Kass, E. H., *ibid.,* 1951, v77, 20.

4. Goldzieher, J. W., Rawls, W. B., and Goldzieher, M. A., *Endocrinol.,* 1953, v52, 580.

5. Lee, N. D., and Williams, R. H., *ibid.,* 1952, v51, 451.

6. Folch, J., Ascoli, I., Lees, M., Meath, J. A., and LeBaron, F. N., *J. Biol. Chem.,* 1951, v191, 833.

7. Lees, M., Ph.D. Thesis, Harvard University, 1951.

8. Letonoff, T. V., and Reinhold, J. G., *J. Biol. Chem.,* 1936, v114, 147.

9. Lurie, M., *Endocrinol.,* 1928, v12, 84.

10. Lieberman, S., Brazeau, P., and Hariton, L. B., *J. Am. Chem. Soc.,* 1948, v70, 3094.

Received March 26, 1956. P.S.E.B.M., 1956, v92.

Secretion of Aldosterone by the Zona Glomerulosa of Rat Adrenal Glands Incubated *in Vitro.** (22416)

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Previous studies(1) have shown that rat adrenal glands incubated *in vitro* release at least seven compounds including a substance with marked sodium retaining activity. The latter compound was identical with aldosterone as far as blue tetrazolium and triphenyl tetrazolium reactions, soda fluorescence, chromatographic mobility, mixed chromatograms and biological activity were concerned. Since the classical work of Deane, Shaw and Greep(2) much indirect evidence has pointed to the adrenal zona glomerulosa as the site of production of mineralocorticoids. The present work provides evidence for the *in vitro* secretion of aldosterone by the zona glomerulosa following the separation of this zone from the rest of the gland by adrenal decapsulation.

Methods. Adrenal glands were obtained from male rats weighing between 170 and 180 g. These animals were subsequently used for bioassay purposes. The glands were carefully dissected free from fat. A small incision was made in the adrenal capsule and the gland was extruded by gentle pressure. In each experiment a total of 50 to 70 glands and their capsules were weighed separately and placed in 50 ml Erlenmeyer flasks containing 1.5 ml per 25 mg of wet tissue of Krebs Ringer bicarbonate solution with added glucose (200 mg %)(3). The incubation of the tissue was carried out in a water bath at 38°C for 2½ hours. After the first hour (pre-incubation period) the medium was replaced by the same volume of fresh Krebs Ringer bicarbonate solution, and the

* This work was supported by consolidated grant from National Research Council of Canada.

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TABLE I. Comparison of Aldosterone Production of Decapsulated with That of Whole Rat Adrenal Glands Incubated *In Vitro*.

Decapsulated glands			Whole glands		
	Aldosterone			Aldosterone	
Tissue wt incubated, mg	μg/total tissue wt /hr	μg/100 mg tissue/hr	Tissue wt incubated, mg	μg/total tissue wt /hr	μg/100 mg tissue/hr
			870	3.22	.37
			868	4.34	.50
1186	.76	.06	875	5.60	.64
1186	.68	.05	733	2.64	.36
527	1.21	.23	750	5.93	.79
1913	.57	.03	798	6.15	.77
528	.66	.13	1006	6.24	.62
1913	.80	.04	730	8.76	1.20
		.09 ± .03			.66 ± .09

incubation was allowed to continue for 90 minutes. Oxygen, containing 5% CO_2, was bubbled through the medium continuously. Incubation media from the glands and from the capsules were each extracted, first with twice and then with 1.5 times their volume of redistilled chloroform. Chloroform extracts were centrifuged to break down the emulsion and evaporated to dryness *in vacuo* at 50°C. Dry chloroform extracts (crude fraction) were chromatographed in the benzene-aqueous methanol system of Bush(4) run at room temperature for four and a half hours. The detection of the steroid material after chromatography was done by direct visualization under ultra violet light, by scanning of the paper strip in a Beckman DU spectrophotometer at 2400 Å; by the soda fluorescence, the blue tetrazolium and the triphenyltetrazolium reactions on the paper chromatogram. The aldosterone band was cut from the paper in the limits of its ultraviolet absorption as defined by the scanning pattern and eluted with redistilled methanol. This fraction was evaporated to dryness, redissolved in ethanol and bioassayed on adrenalectomized rats according to a modification of the Singer-Venning assay(5).

Results. Table I compares the aldosterone production of decapsulated and whole rat adrenal glands incubated *in vitro*. In each series of experiments the aldosterone production is expressed per total weight and per 100 mg of tissue per hour. The whole glands have a mean of aldosterone secretion of 0.66 ± .09 μg/100 mg of tissue per hour, which is in the range of results previously obtained(1), whereas the decapsulated glands only produce 0.09 ± .03 μg of aldosterone per 100 mg of tissue per hour. This difference is statistically significant ($P < .001$).

A typical scanning pattern of two paper chromatograms on which have been developed respectively the extracts of decapsulated gland and of capsule incubates is presented in Fig. 1. In the chromatogram from the capsule there is an obvious aldosterone peak and very little other Δ^4-3-ketosteroid material, whereas in the chromatogram from the decapsulated glands there is no aldosterone peak

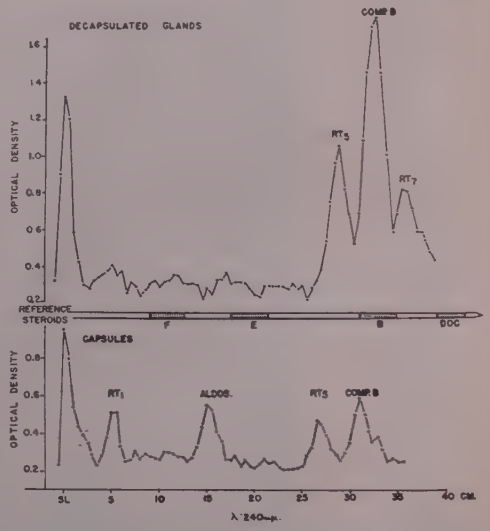

FIG. 1. Location of Δ^4-3-ketosteroids as determined on chromatograms in a calibrated scanner fitted to the Beckman spectrometer. (Wave length: 240 mμ.)

TABLE II. Comparison of Aldosterone Production of Rat Adrenal Capsules with That of the Corresponding Decapsulated Glands Incubated *In Vitro*.

Total wt, mg		Aldosterone				
		Capsules		Decapsulated glands		Whole glands
Capsules	Decapsulated glands	μg/total tissue wt /hr	μg/100 mg tissue/hr	μg/total tissue wt /hr	μg/100 mg tissue/hr	μg/100 mg tissue/hr (calculated)
168	650	4.16	2.46	.48	.11	.71
180	650	4.80	2.66	1.23	.19	.57
244	506	3.54	1.44	1.60	.32	.70
197	542	2.04	.77	1.17	.17	.33
165	547	3.02	1.83	.00	.00	.43
173	569	2.78	1.59	.30	.05	.42
			1.79 ± .28		.14 ± .05	.53 ± .07

and most of the material detected is in the corticosterone area.

In 6 experiments the production of aldosterone, by the decapsulated glands on one hand and by their own capsules on the other, was compared (Table II). The results of the biological assay show that a mean of 1.79 ± .28 μg of aldosterone per 100 mg of tissue per hour is produced by the "capsules," and a mean of 0.14 ± .05 μg 100 mg of tissue hour by the decapsulated glands ($P < .001$). Although values have been given in Table II for the production of aldosterone by the decapsulated gland, in only 2 cases a statistically significant sodium retaining activity was obtained in the bioassay. Thus the average production of aldosterone by the decapsulated gland is probably much lower than that expressed and the difference between the amount produced by the decapsulated gland and by the capsule is even more marked. It should also be noted that the amount of al-

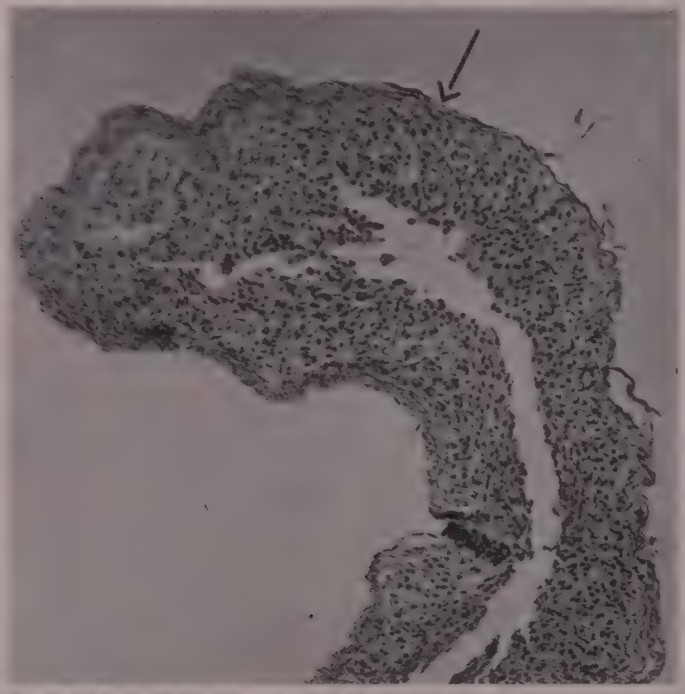

FIG. 2. A Part of a capsule after enucleation of adrenal gland. Zona glomerulosa and outermost part of fasciculata remained attached to the capsule. (× 100, Masson trichrome.)

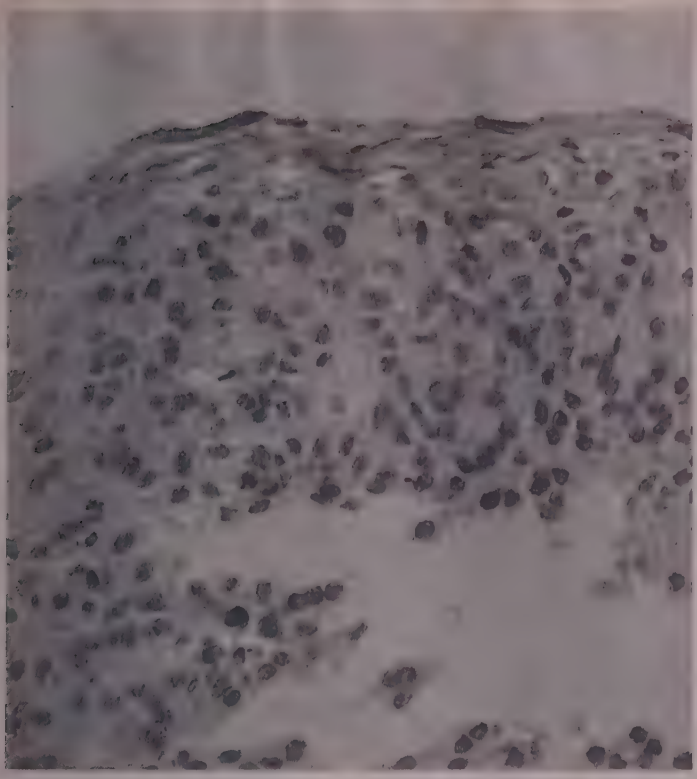

FIG. 2. B) Higher magnification of the area indicated by the arrow on A. (× 425).

dosterone produced by the total tissue mass of the capsule is much greater absolutely than that produced by the total mass of the decapsulated gland ($P<.001$).

The last column of Table II gives the value of aldosterone production per 100 mg of tissue per hour calculated on the basis of the total gland weight (capsule + gland). These values agree well with the amount actually produced by incubated whole glands as shown in the last column of Table I. The difference is statistically not significant ($P>.3$).

Serial histological sections of the adrenal capsules and of the decapsulated glands show that following decapsulation the whole zona glomerulosa and about one-fifth of the zona fasciculata (Fig. 2 A and B) are removed with the connective tissue of the capsule whereas the decapsulated glands contain the rest of the fasciculata, the reticularis and the medulla. Evidence that such a clear cut picture is not always obtained is afforded by the finding of occasional clusters of glomerulosa cells present in the decapsulated portion of the gland. The histological studies have been limited, up to now, to a small number of samples as it would be next to impossible to carry out systematic histological studies on each of the hundreds of the capsules and glands which have been incubated in these experiments. There is no serious reason to believe, however, that a standardised procedure of handling the tissue would lead to a striking difference in the distribution of glandular tissue between the capsular and glandular portion of the adrenal after decapsulation.

Only in 2 instances out of 6 experiments did the decapsulated glands produce enough aldosterone to give a significant sodium retention in the bioassay. Even in these 2 cases the aldosterone production of the "capsules" was more than four times greater than that of the glands when expressed per 100 mg of

tissue per hour of incubation.

Clearly the convention of expressing aldosterone output in terms of tissue weight minimizes the secretory capacity of the decapsulated portion since the non-productive medulla is included in the weight of this part of the gland. It is believed that this consideration does not invalidate the conclusion that aldosterone is produced primarily by the capsular portion and by the zona glomerulosa. This conclusion is based on the following reasons: an arbitrary correction factor which assumes the weight of the medulla to be 50% of the decapsulated gland still yields a significant difference when the corrected output of the decapsulated glands is compared to that of the capsules ($P < .001$). Calculation of aldosterone output in terms of total weight of tissue incubated shows a significantly greater output by the capsules ($P < .001$). Finally as pointed out previously, the aldosterone production of the intact glands (Table I) is strictly comparable to that of the sum of the two portions of the glands incubated separately ($P > .3$), emphasizing the difference in output in favor of the capsule containing the zona glomerulosa. It is therefore concluded that the present findings strongly suggest that aldosterone is produced principally by the cells of the zona glomerulosa.

Conclusion. Rat adrenal glands incubated *in vitro* release aldosterone at the rate of 0.66 μg/100 mg of tissue per hour. Decapsulated rat adrenal glands secrete only negligible amounts of the hormone. Comparison of aldosterone production between decapsulated rat adrenals and their capsules shows that most of the hormone is secreted by the capsular portion which contains the cells of the zona glomerulosa. These findings are strong evidence that the principal site of aldosterone secretion in the rat adrenal is the zona glomerulosa.

We are deeply indebted to Mr. M. Vulpe, Department of Neuro Anatomy, Montreal Neurological Institute, for the histological study and for his valuable advice.

1. Giroud, C. J. P., Saffran, M., Schally, A. V., to be published.
2. Deane, H. W., Shaw, J. H., Greep, R. O., *Endocrinol.*, 1948, v43, 133.
3. Saffran, M., Schally, A. V., *ibid.*, 1955, v56, 523.
4. Bush, I., *Biochem. J.*, 1951, v50, 370.
5. Venning, E. H., Dyrenfurth, I., Giroud, C. J. P., *J. Clin. Endocrinol. & Metab.*, in press.

Received April 9, 1956. P.S.E.B.M., 1956, v92.

Evidence for Occurrence of 10-Formyltetrahydrofolic Acid in Human Urine. (22417)

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The term "citrovorum factor activity" is commonly used to denote the growth-promoting effect of certain substances for *Pediococcus cerevisiae*, formerly known as *Leuconostoc citrovorum* (1,2). Citrovorum factor (CF) activity is shown by 5-formyl-5, 6, 7, 8-tetrahydropteroylglutamic acid (5CHOTHFA) (3,4), by crude preparations of tetrahydropteroylglutamic acid (THFA) (5), and by massive concentrations of pteroylglutamic acid (FA) (1).

The urinary excretion of CF activity in adult males was shown to increase markedly following the oral administration of FA (6,7), thus indicating that FA was converted *in vivo* to CF. However, these studies were based upon microbiological assays in which the samples were autoclaved and hence gave no indication as to whether heat-labile substances with CF activity were present in the urine. Recent studies of the mechanism of CF formation in certain enzyme systems have demonstrated the existence of such heat-labile substances (8,9) thus prompting the con-

TABLE I. Citrovorum Factor (CF) Activity in Urine following Oral Administration of Folic Acid. Values are expressed in terms of DL-5CHOTHFA, 4 samples.

Dose administered		CF excretion in 12 hr		Heat labile CF
Folic acid, mg	Ascorbic acid, g	Heated sample, γ	Unheated sample, γ	% of total CF
10	—	17	36	53
50	—	219	420	48
50	1.0	59	277	78
50	1.0	35	180	81

sideration that human urine might contain such forms of CF. This communication presents evidence from microbiological studies and paper chromatography that a heat-labile form of CF, termed HLCF, exists in human urine. The relationship of HLCF to 10-formyltetrahydropteroylglutamic acid (10CHOTHFA), anhydroleucovorin (AHL) and 5CHOTHFA is discussed.

Methods. CF assays were carried out with *Pediococcus cerevisiae* (*Leuconostoc citrovorum*, ATCC #8081) following the technic of Sauberlich(1). Leucovorin (synthetic DL-5CHOTHFA) was used as the reference standard; results are expressed in terms of the DL anhydrous free acid. Microbiological assays were carried out in 11 x 102 mm test tubes in a final volume of 2 ml. Test samples were either autoclaved with the medium for six minutes, or by contrast were added aseptically in a volume of 0.1 to 0.2 ml to previously autoclaved and cooled medium. For paper chromatography 0.05 ml samples of urine were spotted on one-half inch strips of Whatman #1 paper and developed with 0.1 M sodium acetate, pH 5.4, for 2 hours at 25°C with ascending technic. Bioautography of paper chromatograms was accomplished by placing strips on agar plates of medium which were appropriate for growth of either *L. citrovorum* or *Streptococcus faecalis* (ATCC #8043) and were seeded with an inoculum of the respective organism. Location and size of zones were recorded after the plates were incubated for 16 hours at 37°C. Urine samples were obtained from adults who had taken an oral dose of folic acid and ascorbic acid before retiring. Urine was collected for the next twelve hours, divided into a series of aliquots and stored at −10°C. Aliquots for assay were withdrawn, thawed, and used immediately.

Results. The total amount of CF excreted in 12 hours following the oral administration of FA singly or with ascorbic acid is shown in Table I. Significantly higher excretion values of CF were obtained when the samples were assayed aseptically indicating a possibility that more than one form of CF might be present in the samples. Further evidence for this possibility was obtained by paper chromatography. In many experiments difficulty was encountered in obtaining rigidly reproducible R_f values for the CF forms in urine; this was believed due to the presence of salts in urine and the effects of heating urine. More weight was given to the positions of the growth zones relative to each other rather than to constant R_f values.

Chromatograms of freshly thawed urine showed 2 zones of growth for *L. citrovorum* (Table II). No growth zone appeared at R_f 0.31 if the samples were first subjected to 30 minutes autoclaving. This located the HLCF area on the chromatogram. The zone at R_f 0.7 (± 0.10) coincided with the growth response zone obtained with 5CHOTHFA. The data of Table I indicated that 81% of the CF content of sample #4 was HLCF, yet a qualitative inspection of the bioautograph of the sample (Table II) showed the major form of CF to be 5CHOTHFA as judged by the size of the zone. It is possible that there was some conversion of HLCF to 5CHOTHFA during the course of the experiment, although

TABLE II. Paper Chromatography of Urine and Folic Acid Derivatives. System: 0.1 M acetate (pH 5.4); bioautography with *L. citrovorum*.

Sample	Treatment	pH	R_f and zone size	
Urine	None	6.5	.31 (2+)	.67 (5+)
"	*	6.5	—	.70 (7+)
"	†	2.3	.28 (2+)	.70 (2+)
Leucovorin	†	6.5	—	.76
"	†	2.2	.36	—
Anhydroleucovorin	†	6.5	—	.77 (hazy)
	†	2.2	.43	—

* Autoclaved 30 min. at 120°C.
† Stood 2 hr at 25°C.

it seems more likely that much HLCF was destroyed during the experimental manipulations whereas 5CHOTHFA was stable.

Table II also shows that when urine was incubated at pH 2.3 and subsequently chromatographed, the growth area corresponding to HLCF remained and the 5CHOTHFA zone simultaneously diminished in size. The interpretation of this experiment was complicated by the fact that 5CHOTHFA is converted to AHL under these conditions and could not be distinguished from HLCF in this chromatographic system (Table II). The possibility was considered that HLCF might be AHL; however, it was found that HLCF could be consistently detected in whole urine standing at pH 6.5, whereas, when AHL was held under these conditions, only a slight growth zone could be demonstrated and this zone corresponded to leucovorin (Table II). The finding that AHL at pH 2.2 should support growth of *L. citrovorum* with a clearly discernible zone at 0.43 was unexpected in view of the low microbiological activity of AHL in conventional tube assays(10). Evidently AHL was decomposed during the assay procedure in the earlier investigation(10), presumably during autoclaving. This prompted a study of the effect of pH and reducing conditions on the microbiological activity of AHL for *L. citrovorum*. Table III illustrates that AHL at pH 2.3 had about 13% of the CF activity of leucovorin for *L. citrovorum* and the activity was independent of ascorbate. However, when AHL was incubated at pH 6.5, ascorbate was necessary to maintain biological activity, whether AHL was dissolved in buffer or normal urine. The possibility that HLCF was AHL or a closely related compound stabilized by the ascorbate in the urine is compatible with the findings of Table III.

TABLE III. Lability of Anhydroleucovorin to Air and pH. 100 γ anhydroleucovorin dissolved in 1 ml 0.1 M PO_4 buffer (pH 2.35). Initial activity, 13.3 μg/ml in terms of DL-5CHOTHFA.

Solvent		Conditions during next 2 hr at 27°C 0.1% ascorbate	CF activity as 5CHOTHFA, μg
.1 M PO_4	pH 6.5	Absent	.8
Idem	"	Present	6.4
"	pH 2.35	Absent	12.0
"	"	Present	13.3
Normal urine*	pH 6.5	Absent	2.3
Idem	"	Present	13.0

* From an adult male not dosed with FA and ascorbate.

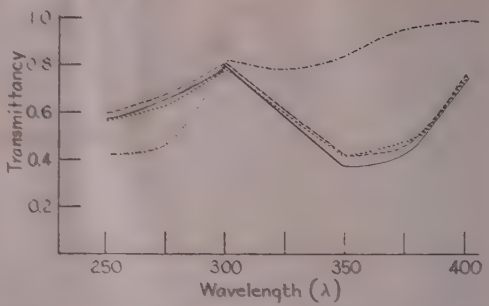

FIG. 1. Ultraviolet absorption spectrum of AHL (0.1 M phosphate buffer) solutions diluted to 10 γ/ml in 0.01 N HCl just prior to reading in a model DU Beckman spectrophotometer. Curves: ———, AHL in pH 2.3 buffer, 2 min. at 25°C; · · · · ·, AHL in pH 2.3 buffer after 2 hr at 25°C; – – – –, AHL in pH 6.5 buffer, 2 min. at 25°C; •—•—•—, AHL in pH 6.5 buffer after 2 hr at 25°C.

Spectrophotometric analyses of solutions of AHL in 0.1 M phosphate buffer at pH 2.2 and 6.5 were made at zero and 2 hours. Fig. 1 shows that a loss in absorption between 350 and 360 mμ occurred at pH 6.5 after 2 hours, showing a chemical change, but no spectral change was observed at pH 2.2. An observable chemical change thus accompanied the loss of microbiological activity of AHL at pH 6.5.

An attempt was made to purify HLCF by large scale paper chromatography to permit a more detailed study of its chemical relationship to other FA derivatives. HLCF and FA had approximately identical R_f values in the solvent system employed. The high concentration of FA in the urine used as a source of HLCF however, was not a problem in these studies because the location of HLCF on chromatograms was determined by bioautography with *L. citrovorum* which was insensitive to the amount of FA in the aliquot. Aliquots of 0.05 ml of urine containing HLCF were spotted along a line 1" from the lower edge of a Whatman #1 paper sheet (18½ x 11½). The chromatographic technic used was similar to that described under *Methods*.

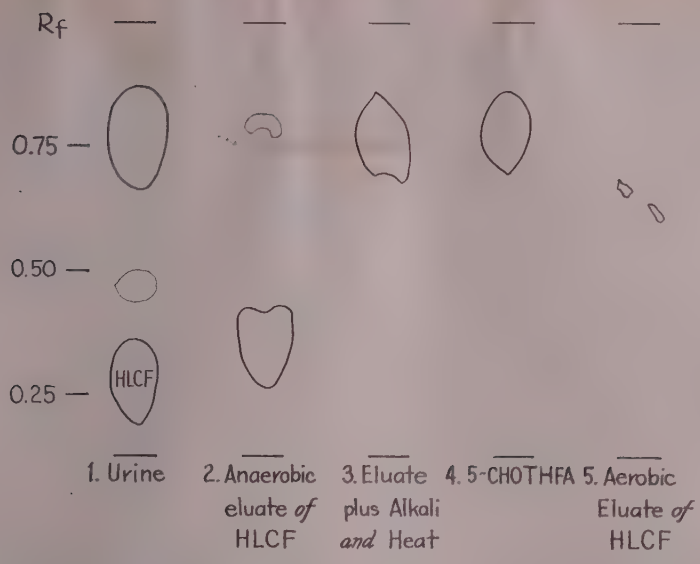

FIG. 2. Evidence for conversion of HLCF to 5CHOTHFA. Chromatography system; 0.1 M sodium acetate, pH 5.4; bioautography with *L. citrovorum*. 1. Urine; 2. Anaerobic eluate of HLCF; 3. Anaerobic eluate adjusted to 0.1 N alkali and heated at 100°C; 4. Leucovorin; 5. Aerobic eluate of HLCF.

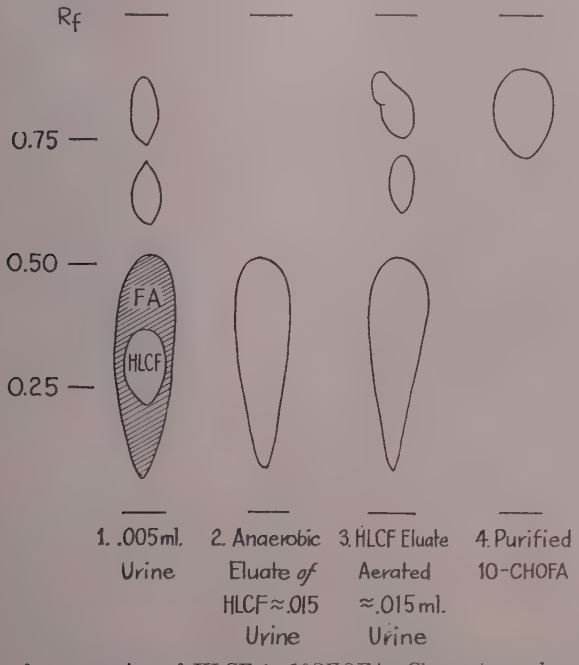

FIG. 3. Evidence for conversion of HLCF to 10CHOFA. Chromatography system; 0.1 M sodium acetate, pH 5.4; bioautography with *S. faecalis*. 1. 0.005 ml urine; 2. Anaerobic eluate of HLCF = 0.015 ml urine; 3. Aerated eluate of HLCF = 0.015 ml urine; 4. purified 10-CHOFA.

The HLCF area on the chromatogram, predetermined by bioautography of a ½" vertical section was cut out and cut into smaller squares which were stacked in a small pyrex funnel. An appropriate buffer was then applied to the paper with a pipette. The volumes of eluant were about twice the original volume of urine.

When the HLCF area was eluted with 0.1 M phosphate buffer, pH 6.5, and aerated for one hour, HLCF activity could not be demonstrated (strip #5, Fig. 2), but it could be shown that such "aerobic" eluates contained a substance (R_f 0.78-0.80) active for growth of *Streptococcus faecalis* (Strip #3, Fig. 3) and this factor could not be distinguished in 3 solvent systems from a sample of 10CHOFA, purified by the method of Silverman (11). This experiment indicated the presence of a formyl group in HLCF.

When the HLCF area was eluted with 0.1 M phosphate pH 6.5 buffer with the addition of 0.1% ascorbate to bring about anaerobic conditions, HLCF could be recovered from paper chromatograms (strip #2, Fig. 2). These observations emphasized the highly reduced state of HLCF. When such an anaerobic eluate was made alkaline (0.1 N) and heated at 100°C under nitrogen for one hour (3), 5CHOTHFA was formed as indicated by Fig. 2, strip 3, leading to the conclusion that HLCF is a formyl derivative of tetrahydrofolic acid.

Discussion. The chemical changes pertinent to the present investigation are shown in abbreviated form in Fig. 4. The chemical studies of May *et al.*(4) showed that AHL presumably exists only in acid solution, and that when such a solution is made neutral or alkaline the imidazoline ring is ruptured and 10CHOTHFA is formed provided that air is excluded from the solution. Moreover, 10CHOTHFA presumably does not exist in acid solutions for under these conditions it is transformed into AHL. The following lines of evidence support the conclusion that HLCF present in human urine under the conditions described herein was 10CHOTHFA: (a) HLCF was stable at a neutral pH in the presence of ascorbic acid, (b) under aerobic conditions HLCF was converted to 10CHOFA, and (c) under anaerobic conditions when HLCF was made alkaline and heated 5CHOTHFA was readily formed. These properties of HLCF correspond to those of 10CHOTHFA which are shown in Fig. 4. Similar evidence has been obtained to establish the identity of 10CHOTHFA formed from THFA by enzyme systems from pigeon liver and pig heart(12,13). The present study demonstrates that 10CHOTHFA is formed in man.

Nichol and coworkers(8) recently described the enzymatic formation of a labile precursor of CF, termed "CFX", from FA by several enzyme systems. "CFX" was converted non-enzymatically to CF by heating and it was labile to oxygen; in these respects "CFX" resembles HLCF. However HLCF possesses microbiological activity for *L. citrovorum* prior to heating. The fact that a form of CF having an R_f indistinguishable from 5CHOTHFA exists in fresh unheated human urine implies that 5CHOTHFA may arise by

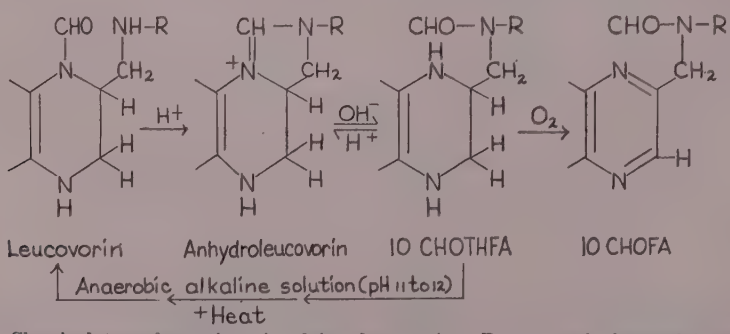

FIG. 4. Chemical transformations involving leucovorin; R = p-aminobenzoylglutamic acid. Pyrimidine ring is omitted to save space.

physiological processes as well as by chemical transformation from 10CHOTHFA-like compounds. Studies by Kisliuk and Sakami(14) and Blakley(15) have established the existence of a form of CF in which the one carbon functional group is at the formaldehyde level of oxidation (5 or 10 hydroxymethyl-THFA, or the corresponding 5-10 bridge compound). It seems unlikely that HLCF is a hydroxymethyl derivative since such a compound would have to undergo an oxidative step before chemical conversion to 5-CHOTHFA.

While this work was in progress we were informed(16) that Silverman has also demonstrated the existence of a labile derivative of CF in human urine and has reached conclusions similar to ours regarding its chemical nature.

Summary. Microbiological studies with *Leuconostoc citrovorum,* ATCC 8081 (*Pediococcus cerevisiae*) showed that a heat labile form of citrovorum factor (HLCF) occurs in the urine of normal adults after an oral dose of folic and ascorbic acids. Anhydroleucovorin (AHL) resembled HLCF in that AHL was active for growth of *L. citrovorum* (13% as active as leucovorin) when acid solutions were assayed aseptically, but neutral solutions of AHL were low in microbiological activity unless ascorbic acid was present. HLCF was purified by large scale paper chromatography which permitted a study of its chemical properties. The chemical relationships between 5CHOTHFA, AHL, and 10-CHOTHFA were discussed with regard to the chemical properties that were demonstrated for HLCF from which it was concluded that HLCF is probably 10CHOTHFA.

The authors wish to thank Dr. Thomas H. Jukes for his interest and encouragement in this investigation and for his helpful suggestions and criticisms in the preparation of this manuscript.

1. Sauberlich, H. E., and Baumann, C. A., *J. Biol. Chem.,* 1948, v176, 165.

2. Felton, E. A., and Niven, C. F., *J. Bacteriol.,* 1953, v65, 482.

3. Roth, B., Hultquist, M. E., Fahrenbach, M. J., Cosulich, D. B., Broquist, H. P., Brockman, J. A., Jr., Smith, J. M., Jr., Parker, R. P., Stokstad, E. L. R., and Jukes, T. H., *J. A. C. S.,* 1952, v74, 3247.

4. May, M., Bardos, T. J., Barger, F. L., Lansford, M., Ravel, J. M., Sutherland, G. L., and Shive, Wm., *ibid.,* 1951, v73, 3067.

5. Broquist, H. P., Fahrenbach, M. J., Brockman, J. A., Jr., Stokstad, E. L. R., and Jukes, T. H., *ibid.,* 1951, v73, 3535.

6. Anker, R. M., Boehne, J. W., and Welch, A. D., *Fed. Proc.,* 1950, v9, 351.

7. Broquist, H. P., Stokstad, E. L. R., and Jukes, T. H., *J. Lab. Clin. Med.,* 1951, v38, 95

8. Nichol, C. A., Anton, A. H., and Zakrzewski, S. F., *Science,* 1955, v121, 275.

9. Deodhar, S., Sakami, W., and Stevens, A. L., *Fed. Proc.,* 1955, v14, 201

10. Cosulich, D. B., Roth, B., Smith, J., Jr., Hultquist, M. E., and Parker, R. P., *J. A. C. S.,* 1952, v74, 3252.

11. Silverman, M., Keresztesy, J. C., and Koval, G. J., *J. Biol. Chem.,* 1954, v211, 53.

12. Jaenicke, L., *Biochimica et Biophysica Acta,* 1955, v17, 588.

13. Greenberg, J. Robert, Jaenicke, L., Silverman, M., *ibid.,* 1955, v17, 589.

14. Kisliuk, R. L., and Sakami, W., *J. Biol. Chem.,* 1955, v214, 47.

15. Blakley, R. L., *Biochem. J.,* 1954, v58, 448.

16. Silverman, M., personal communication.

Received April 13, 1956. P.S.E.B.M., 1956, v92.

Thyroxin as Sensitizing Agent in Production of Renal and Cardiovascular Lesions with Corticoids.* (22418)

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Soon after it had been discovered that desoxycorticosterone acetate (DOCA) can produce extensive hyalinization of mesenchymal structures, with nephrosclerosis, myocarditis and periarteritis nodosa in the rat, it became evident that certain "conditioning factors" (*e.g.*, unilateral nephrectomy, dietary sodium supplements) are capable of facilitating the development of this syndrome. Concurrent treatment with other hormones can also modify the production of hyalinosis by DOCA and related prophlogistic corticoids. For instance, an antiphlogistic hormone, such as cortisol acetate (COLA), greatly aggravates the renal changes, but inhibits the purely inflammatory manifestations (especially the periarteritis and, to a lesser extent, the myocarditis) of DOCA-overdosage. Crude anterior pituitary extracts, or partially purified somatotrophic hormone (STH) preparations, exacerbate all the manifestations of the DOCA-induced hyalinosis syndrome—indeed they can produce hyalinosis even without concurrent DOCA-treatment—but only in the presence of functioning adrenal tissue. It had therefore been assumed that STH (or some other anterior pituitary principle which contaminates all available STH preparations) induces hyalinosis through the intermediary of the adrenal cortex, perhaps by enhancing the metabolic transformation of DOCA-like corticoids into compounds still more active in producing hyalinosis. The rather voluminous literature concerning the hormonal factors which influence hyalinosis has been discussed elsewhere(1-6).

Thyroxin shares with STH the ability to sensitize the rat to the production of hyalinosis by DOCA, but it was not known whether in this respect, its effect—like that of STH—is dependent upon the presence of adrenal tissue. It also remained to be shown whether the particularly nephrotoxic effect of combined treatment with DOCA and COLA can be further enhanced by concurrent thyroxin-administration. The experiments to be reported below were performed in order to clarify these points.

Materials and methods. Sixty female Sprague-Dawley rats, with an initial bodyweight of 100-114 g (avg 107 g) were subdivided into 6 equal groups for treatment, as indicated in Table I. Both steroids† were administered subcutaneously in the form of microcrystals, the total daily dose being suspended in 0.2 ml of water (containing the usual suspending agents). Thyroxin‡ was given as the sodium salt, also in 0.2 ml of water, subcutaneously. All animals were conditioned for the nephrotoxic effect of the hormones by the removal of the right kidney on the day hormone-treatment began. At the same time, in the last 2 groups of this series, the adrenals were extirpated through the lumbar route. All the animals received 1% NaCl as a drinking fluid and "Purina Fox Chow" as the sole source of nourishment. In Table I the mean intensity of cardiac and renal lesions, as well as the degree of fluid-accumulation in pleura and peritoneum, are expressed in a scale of 0 to +++. The fluid-intake listed is the mean of the volume ingested during the last 3 days. The experiment was terminated on the 13th day of hormone-treatment.

Results. As indicated in Table I, thyroxin greatly enhanced the production of cardiac and renal lesions, as well as the accumulation of fluid in the large body-cavities. This effect of the thyroid hormone was quite evident, both at the low (*cf.* Groups 1 and 2)

* These experiments were performed with aid of a grant from Gustavus and Louise Pfeiffer Foundation, and grant from the Warner-Chilcott Laboratories.

† Cortisol acetate ("Cortril") was generously supplied by Pfizer Laboratories, and desoxycorticosterone acetate ("Cortate") by Schering Corp.

‡ Obtained from British Drug Houses Ltd.

TABLE I. Sensitization to Certain Corticoid-Overdosage Effects by Thyroxin.

Group	Treatment in μg/day COLA	DOCA	Thyroxin*	Adrenal	Body-wt loss (in %)	Heart-lesions	Kidney-lesions	Ascites and pleural fluid	Fluid-intake (ml/d)	Mortality (in %)
1	500	500	0	Intact	2	0	0 to +	0	87	0
2	500	500	250	"	0	2+	3+	2+	130	20
3	1000	1000	0	"	11	+	+	0	130	0
4	1000	1000	250	"	5	2+	3+	3+	123	30
5	500	500	250	Adr-X	13	2+	3+	+	110	30
6	1000	1000	250	"	19	2+	3+	3+	139	30

* Dose raised to 500 μg during last 2 days of experiment.

and at the high (*cf.* Groups 3 and 4) level of steroid-treatment, and it was not prevented by complete adrenalectomy (*cf.* Group 2 with 5 and Group 3 with 6).

Histologically, the cardiac lesions were mainly characterized by formation of hyalinized foci, surrounded by inflammatory cells, and by periarteritis nodosa of the heart vessels. In the kidney, there was hyalinization of the glomeruli, occasionally accompanied by hemorrhages into Bowman's capsule. Many tubules were dilated and contained more or less intensely hyalinized protein-casts. Conversely some proximal convolutions were completely collapsed; most of these lost their brush-border and their eosinophilia, but often they contained clear cells in mitotic division. This aspect is quite characteristic of the "endocrine nephrons" seen in kidneys whose excretory function is abolished by constriction of the afferent vessels (Fig. 1). Outside the heart, periarteritis nodosa was not observed in any groups of this experimental series, presumably because of the high dose of anti-inflammatory hormone given.

In addition to the fluid-accumulations in peritoneum and pleura, an enormous and generalized subcutaneous edema had developed in all the rats of Group 4 and, to a lesser extent, in a few of the animals in Group 2. No such edema was seen in any of the adrenalectomized animals receiving the same hormone-treatment, although the cardiac and renal lesions were just as severe here as in the presence of the adrenals.

Discussion. The very acute renal and cardiac changes accompanied by pronounced water-retention are rather reminiscent of the "eclampsia-like syndrome," which results when renin is administered to rats pretreated with DOCA(7,8). The latter condition has repeatedly been compared with clinical pre-eclampsia and eclampsia(8,9). Without entering into a discussion of the possible relationship between these experimental and clinical syndromes, it may be pointed out that the changes observed in our rats strikingly resemble those obtained by combined DOCA and renin-treatment. Furthermore, several of the animals in our Group 4 showed nervous manifestations (convulsions, tics), brain-edema and multiple hemorrhages (especially in the heart, kidney, brain and, occasionally, in the peritoneum) all of which have also been reported to occur in the DOCA + renin syndrome. The latter had been thought to be rather specifically due to renin. In rats (similarly conditioned by unilateral nephrectomy, NaCl and pretreatment with DOCA) this "eclampsia-like" condition has also been elicited with hypertensin—the product of enzymatic interaction between renin and its substrate—but not with adrenaline, vasopressin, thromboplastin, ACTH, histamine or crude human placental extracts(8).

The corticoid + thyroxin syndrome described here appears to differ from the DOCA + renin syndrome only in that the former, unlike the latter, does not lead to complete anuria (except perhaps during the last few hours of life). However, in view of the many resemblances, we are nevertheless inclined to regard the 2 syndromes as essentially identical in their manifestations. This does not necessarily invalidate the conclusions of Masson and his colleagues(7,8) concerning the specific pathogenic rôle played by the renal-pressor mechanism. In our rats the many

"endocrine nephrons" might well have supplied an endogenous source of renin or of some related substances.

Summary. In rats, conditioned by unilateral nephrectomy and a high NaCl-intake, 12 days of treatment with comparatively small doses of desoxycorticosterone acetate (DOCA) and cortisol acetate (COLA) causes only very mild renal and cardiac lesions, which result in no detectable fluid-accumulation or mortality. Concurrent administration of thyroxin greatly aggravates the cardiac and renal

FIG. 1. Sensitization by thyroxin for the production of renal and cardiovascular lesions with corticoids. *A.* Control rat of Group 3 treated with COLA and DOCA. Renal structure is essentially normal at the end of this short-term experiment. *B.* Kidney of a rat from Group 4, which was treated with COLA, DOCA and thyroxin. Note intense renal damage, due to hyalinization of the glomeruli and their afferent arterioles. Some of the tubules are dilated, due to obstruction by hyalin-casts, while others have become solid epithelial cords, reminiscent of the nephrons in the "endocrine kidney." *C.* Region from heart of the animal shown in Fig. B. Largely hyalinized granulomatous nodule. *D.* Other region of heart shown in Fig. C. Several small arteries undergoing hyalin-necrosis.

lesions; at the same time it produces pronounced fluid-accumulations in the peritoneum, pleura and subcutaneous tissues, with multiple hemorrhages, nervous disturbances and a high mortality. The syndrome is regarded as essentially similar to that previously produced by other investigators through combined treatment with DOCA and renin. The ability of thyroxin to produce cardiac and renal damage under these conditions—unlike the comparable effects of STH—is not prevented by adrenalectomy.

1. Selye, H., *Stress. The Physiology and Pathology of Exposure to Stress,* Acta Inc., Med. Publ., Montreal, 1950.

2. ———, First Annual Report on Stress, Acta Inc., Med. Publ., Montreal, 1951.

3. Selye, H., and Heuser, G., Fourth Annual Report on Stress, Acta Inc., Med. Publ., Montreal, 1954.

4. ———, Fifth Annual Report on Stress, M. D. Publ., Inc., New York, 1955/56.

5. Selye, H., and Horava, A., Second Annual Report on Stress, Acta Inc., Med. Publ., Montreal, 1952.

6. ———, Third Annual Report on Stress, Acta Inc., Med. Publ., Montreal, 1953.

7. Masson, G. M. C., *Atti d. Soc. lomb. di sc. med. e biol.,* 1954, v9, 348.

8. Page, E. W., and Glendening, M. B., *Am. J. Obst. & Gynec.,* 1955, v69, 666.

9. Alvarez, R. R. de, *ibid.,* 1955, v69, 675.

Received April 26, 1956. P.S.E.B.M., 1956, v92.

Influence of 9-Alpha Fluorohydrocortisone on Contractility and Na Content of Isolated Cat Papillary Muscle.* (22419)

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The effect of steroids upon tissues other than the components of the pituitary-adrenal and renal axis has been studied by many workers, including Ingle, Szego and Eisenberger(1-3). Previous studies(4,5) have shown that under certain conditions progesterone and cortisone exert a negative inotropic action (*i.e.* contractility) on the heart. With a simple electronic system perfected in this laboratory, we have measured a previously unreported inotropic effect of 9-alpha fluorohydrocortisone‡ (9-a FF) on isolated cat papillary muscle. This synthetic hormone was chosen for this study because of its marked mineralocorticosteroid and glucocorticosteroid activity(6).

Methods. The cat papillary muscle preparation employed was that first described by Cattell and Gold(7). Cats anesthetized with ether were killed by cardiectomy. At least 2 and sometimes 3 papillary muscles, varying from 2-15 mg in weight, were removed from the right ventricle. These muscles were individually dissected, leaving the chorda tendinae and some valvular tissue attached to one end, and a tab of myocardium to the other. The papillary muscle was thus isolated without causing tissue injury. A hook, which also served as one electrode on a muscle holder, was placed through the chorda tendinae. From the other end of the muscle a suture attached to the papillary:myocardial juncture was tied to an extension on the pin of an electronic transducer tube (RCA 5734). The transducer tube transformed slight mechanical displacements into electrical potentials which were in turn picked up by a direct-writing Sanborn EKG instrument. A control and a treated muscle were stimulated in two identical chambers which were attached to the electronic system in such a manner that both control and experimental observations could be recorded simultaneously.

* This project was partially supported by funds from Ciba Research Foundation, U.S.P.H.S., Boettcher Foundation and the Continuing Research Fund of University of Colorado School of Medicine.

† U.S.P.H.S. Fellow of the National Heart Institute.

‡ Acknowledgment is made to Merck & Co., Rahway, N. J., which provided us with the 9-alpha fluorohydrocortisone.

TABLE I. Results Obtained with 9-alpha fluorohydrocortisone.

Exp.	Drug conc. (μg/ml)	Counts/sec./ml buffer	Counts/sec. G.	Ratio:* Exp./Cont.	Δ inotropic action (%)†
	Control	1000	246		
1	.625	"	440	1.8	+ 83.7
2	.625	"	480	2.0	+ 56.2
	Control	"	256		
3	.625	"	2955‡	11.0	+ 36.8
4	.50	"			+102.2
	Control	2000	916		
5	.50	"	1172	1.3	+154.5
	Control	"	685		
6	.50	"	1276	1.9	+117.6
	Control	"	705		
7	1.00	"	708	1.0	+ 12.5
	Control	"	236		
8	0.5	"	629	2.6	+136.0
9	.625	"	360	1.5	+168.5
	Control	"	356		
10	.625	"	1040	2.9	+ 46.5
	Control	3000	695		
11	.50	"	2753	3.9	+ 76.0

* In counts/sec./G.

† Measured from just prior to the administration of drug to the highest point reached after administration.

‡ Probable experimental error.

Krebs-Ringer bicarbonate solution enriched with dextrose was used as the perfusing medium. The perfusing solution in which both the control and experimental muscles contracted contained 0.1 ml liter of carrier-free Na^{22} (half-life 2.6 years).§ A mixture of 95% O_2 and 5% CO_2 was bubbled through the buffer by means of a sintered glass filter fixed to the base of the muscle chambers. The muscles were stimulated throughout the experiment at 60 times per minute and maintained at a constant temperature of 38°C and recordings taken at intervals. 9-α FF in small amounts (0.5-1.0 μg ml as indicated in Table I), was added to the buffer bathing one of the muscles. At the conclusion of an experiment, the muscles were carefully dissected from the chorda tendinae and myocardial tab, washed and weighed rapidly then digested in H_2SO_4 and the total radioactivity determined in a well counter. The uptake of isotopic Na was expressed in counts sec. G. and then in terms of the ratio of radioactivity of the samples treated with 9-α FF to that of the control samples.

Results. Fig. 1 shows the effect of adding 9-α FF to the perfusing fluid of 7 muscles, (see Table I, expt. 1-7). The average values of these 7 experiments are plotted in terms of % height of the initial contraction and were measured at intervals throughout the experiments. The broken line represents data from the treated samples and indicates a marked positive inotropic effect on comparison with mean control values represented by the solid line.

Results from individual experiments are tabulated in Table I. The positive inotropic effect is demonstrated in each experiment upon the addition of 9-α FF. Furthermore, the Na^{22} content of each treated muscle was increased on comparison with its control from the other chamber.

Discussion. These data are presented as evidence that marked positive inotropic activity is exhibited by a steroid substance, other than the glycosides or aglycones. In addition, it has been shown that under the influence of 9-α FF, measurable changes in isotopic sodium content take place in the contracting muscle. The possibility that the in-

§ Specific activity = 496 mc G., (<0.02 μg ml); contaminating Na = 0.52γ liter; (a negligible quantity in a buffer containing 140 mEq L. of sodium).

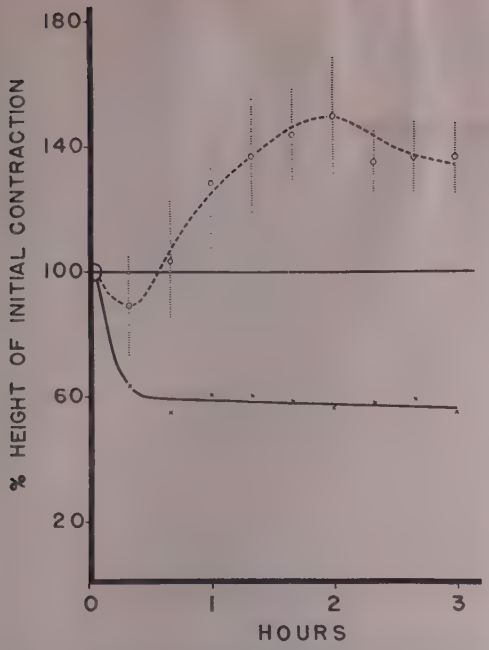

FIG. 1. Upper curve shows positive inotropic action when 9-a FF is added. Vertical lines represent calculated stand. error. Lower curve shows the mean of 11 control experiments.

crease in counting rate of muscles treated with 9-a FF might represent an exchange was considered. However, it does not seem likely that the concentration of intracellular sodium in heart muscle, which has been reported to be from 13.7 to 22.9 mM/K (rat)(8), could show an apparent rise to values which in some cases are over ½ that of the buffer (140 mEq./L.), by a process of exchange alone. Unfortunately, we were unable to obtain direct chemical measurements on the sodium uptake because of the minute size of the muscles studied. It is possible that part, or all of this increase in the treated muscles is a reflection of a marked rise in the intramuscular extracellular fluid. The high sodium content of the controls is in agreement with the observation by Reiter(8) that exercise increases the sodium content of heart muscle. In addition, Flückiger and Verzár(9) have presented evidence that steroid effects on glucose metabolism may be associated with the regulation of muscle electrolyte content. Present work in this laboratory is aimed at establishing the microanatomic site of this change in electrolyte distribution and to decide whether this change represents the cause or the effect of the reported positive inotropic action.

Summary. (1) A new method is described for quantitating inotropic effects in the cat papillary muscle preparation. (2) When 9-alpha fluorohydrocortisone (9-a FF) is added to the perfusate in a concentration of 0.5 to 1.0 μg/ml there is observed a direct effect on the muscle as manifested by a marked positive inotropic action and an increased uptake of radioactive Na.

We wish to thank members of the Radioisotope Service of the Denver V. A. Hospital for their technical advice and the use of their counting equipment.

1. Ingle, D. J., *et al.*, PROC. SOC. EXP. BIOL. AND MED., 1953, v83, 537; *Acta Endocrinol.*, 1953, v14, 93; *Endocrinol.*, 1953, v53, 582.

2. Szego, C., *Recent Progress in Hormone Research*, 1953, v8, 419.

3. Eisenberger, E., and Gordon, G., *J. Pharm. & Exp. Therap.*, 1950, v99, 38.

4. Rubin, B., and Salter, W. T., *ibid.*, 1952, v105, 443.

5. Booker, W. M., DaCosta, F., Mitchell, S. Q., and Robinson, E. J., *ibid.*, 1956, v116, 8.

6. Fried, J., *Ann. N. Y. Acad. Sci.*, 1955, v61, 573.

7. Cattell, McK., and Gold, H., *J. Pharm. & Exp. Therap.*, 1938, v62, 116.

8. Reiter, M., *Arch. f. exp. Path. u. Pharm.*, 1955, v225, 138.

9. Flückiger, E., and Verzár, F., *Acta Endocrinol.*, 1954, v17, 80.

Received January 16, 1956. P.S.E.B.M., 1956, v92.

Influence of Gentling on Physiology of the Rat. (22420)

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It appears that various physiological processes in the albino rat can be altered by handling or gentling the animal for a prescribed time each day. It has been shown that gentled rats survive certain surgical procedures better than non-gentled animals(1-3) and that gentled animals possess greater discrimination learning capacity(4). Both the Weininger group(5,6) and our group(7) have studied effects of gentling on growth, thyroid metabolism and ability to withstand certain forms of stress. Weininger found that rats grew better and survived prolonged starvation better than non-gentled controls. We also found a relationship between gentling and growth, and attempted to enhance differences between gentled and non-gentled rats by subjecting animals to stress of starvation or electrical shock.

Methods. Sprague-Dawley weanling male rats weighing 40-44 g were distributed at random into groups of 8 to 12 rats each. All animals were housed in individual galvanized iron cages having wire mesh bottoms, and were fed commercial rat food* and water *ad libitum*. Weekly records were kept of individual weight gains and food and water consumptions. All animals from the gentled groups were removed from cages for 10 min. each day and were fondled individually by an investigator. At the conclusion of each experiment, all animals were injected intraperitoneally with 1 ml of I-131 carrier-free iodide (50 μc), and the animals sacrificed and autopsied 24 hr later. The carcasses were suspended by the tail, and measurements of length were made from tip of nose to anus. Thyroid glands were removed *in toto* and digested with NaOH until completely hydrolyzed. Aliquots were counted in a Nancy Wood Scintillation Well Counter to standard deviation of 2%, and percent dose calculations were made by comparing these counting rates with a 1:1000 dilution of standard dose. The shocking device consisted of induction coil connected to copper wire grid threaded into bottom of small cardboard box. The animal was held securely by tight fitting lid, and repeated short electrical shocks were administered for 10 min. Entire carcasses (except tails) of all animals in Exp. 1 and 2 were ground to homogenous mixture in meat grinder. Samples were analysed for total moisture, neutral fat and Kjeldahl nitrogen. Arithmetic means, standard deviations and errors were calculated, and a Student t test was made of significance of differences. A p value of .02 or less was considered to be significant.

Results. Twelve rats in each group were employed in first 2 experiments, and 12 and 9 rats respectively in single and grouped tests of Exp. 3. A summary of data is given in Table I. Gentled animals, whether individually caged or grouped, showed consistently and significantly better growth and food utilization values than non-gentled animals. Thyroid I-131 uptake measurements were always lower for gentled animals, although the significance of these differences was questionable. On the other hand, neither gross pathological disturbances nor differences in moisture, fat and nitrogen values for gentled and non-gentled animal carcasses could be found.

An experiment was carried out with 4 groups of 12 animals each, fed food and water *ad libitum* for 1 wk. after weaning. Two of these groups were gentled in the usual manner, the remaining 2 were not. At the end of this time, one gentled and one non-gentled group was placed on moderately restricted food intake of 8 g/day for first wk. and 10 g/day thereafter. The other gentled and non-gentled groups were alternately subjected to complete starvation for 48 hr and recovery. Usual growth and food utilization differences between gentled and non-gentled animals were evident for the first wk., but

* GLF Service, Syracuse, N. Y.

TABLE I. Summary of Physiological Differences Observed between Gentled and Non-Gentled Animals.

		Groups				Compare	
	Test No.	G-SC	NG-SC	G-DC	NG-DC	G-SC *vs* NG-SC	G-DC *vs* NG-DC
		Arithmetic means				p values	
Wt gain (g)	1	187.2	169.8			.007	
	2	189.9	154.3	163.0	145.5	<.001	.026
	3	155.7	145.1	153.6	143.7	.04	.05
Food utilization (g)	1	3.62	3.98			.008	
	2	3.28	3.65	3.40	3.76	<.001	.002
	3	3.31	3.64	3.40	3.63	"	.08
Carcass length (cm)	1	19.99	18.90			<.001	
	2	19.48	18.80	19.68	18.51	"	<.001
	3	20.27	19.56	20.30	19.55	"	"
Thyroid uptake (% dose)	1	9.40	9.65			.75	
	2	2.60	3.80	2.99	3.22	.005	.3
	3	2.80	3.07	2.67	2.72	.03	.8

G and NG designate gentled and non-gentled animals. SC and DC designate single caged and double caged animals.

these were essentially irradicated during food restriction or starvation (Table II, Fig. 1).

A further experiment was carried out in which animals were gentled for 2 wks. prior to stress. Then 8 rats from each group of gentled and non-gentled animals were sacrificed and carcass lengths and thyroid uptakes measured. Remaining animals were divided into the following groups of 8 rats each: gentled-shocked, non-gentled-shocked, gentled-starved, non-gentled-starved and non-gentled controls. Growth and food utilization were measured for the next 2-3 wks., then animals were sacrificed, autopsied and carcass length and thyroid uptakes were measured. Data are summarized in Table III and Fig. 2 and 3. With respect to starved animals, there were consistent differences between weight gains, thyroid uptake values and carcass lengths of gentled and non-gentled groups. Non-gentled-starved animals were unquestionably inferior to non-gentled controls in all categories. In shocked groups, gentled animals lost their initial advantage, and eventually attained similar weight gains as non-gentled group (Fig. 3). Therefore, weight gains and food utilization figures for gentled animals during the second 2 wk. period were relatively inferior.

Discussion. The first 3 experiments demonstrate that gentled animals grow better, utilize food more efficiently for weight gain, and tend to exhibit lower thyroid I-131 uptake measurements than non-gentled controls. Although the thyroid differences are too small to be statistically significant, they are consistently in the same direction and suggest that gentling may lower basal metabolism of

TABLE II. Summary of Physiological Differences Observed between Gentled and Non-Gentled Animals Subjected to Food Restriction or Electrical Shock for 3 Weeks.

	Groups				Compare	
Measurement (at 4 wk)	G-R	NG-R	G-St	NG-St	G-R *vs* NG-R	G-St *vs* NG-St
	Arithmetic means				p values	
Wt gain (g)	90.4	96.1	77.8	76.0	.08	.78
Food utilization (g)	3.32	3.16			.17	
Carcass length (cm)	17.90	18.31	18.1	17.80	.01	.07
Thyroid uptake c food (% dose)	27.9	22.17	12.0	17.2	.01	.02
s food			25.9	25.0		.74

G and NG designate gentled and non-gentled animals. R and St designate restricted food intake and starved animals.

TABLE III. Comparison of Growth, Food Utilization, Carcass Length, and Thyroid Uptake Values of Gentled and Non Gentled Animals under Stress of Starvation or Shock.

	Second 2 weeks			Second 3 weeks			Compare			
	G-Sh	NG-Sh	NG-C	G-St	NG-St	NG-C	G-Sh *vs* NG-Sh	NG-Sh *vs* NG-C	G-St *vs* NG-St	NG-St *vs* NG-C
	Arithmetic means						p values			
Wt gain (g)	50.0	63.6	85.2	55.0	47.3	113.2	.005	<.001	.05	<.001
Food utilization (g)	5.6	4.3	3.32			3.36	.001	"		"
Carcass length (cm)	18.97	19.28		18.72	17.78	20.17	.01		.004	
Thyroid uptake (% dose)	24.1	19.9		14.28	17.53	12.03	.13		.07	.02

G and NG designate gentled and non-gentled animals. Sh, St, and C designate shocked, starved and control animals respectively.

FIG. 1. Effect of pre-gentling for 1 wk on growth of animals subjected to starvation.

the rat by influencing the amount of thyroid hormone produced. This might be envisioned as alteration in amount of stimulation received by the hypophysis from the hypothalamus, which might in turn alter the amount of thy-

FIG. 2. Effect of pre-gentling for 2 wk on growth of animals subjected to starvation.

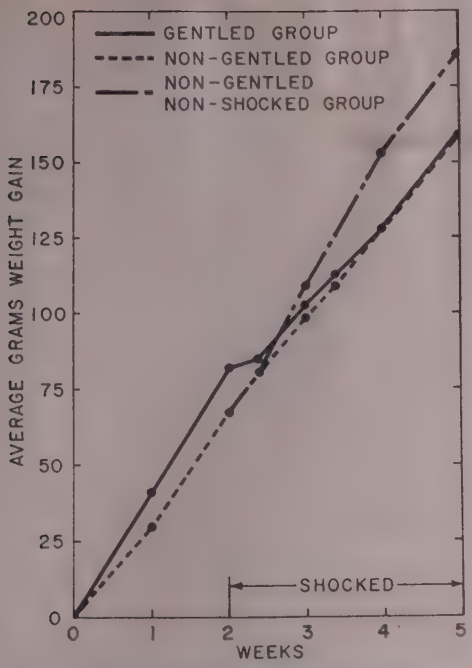

FIG. 3. Effect of pre-gentling for 2 wk on growth of rats subjected to electrical shock.

roid stimulating hormone produced by the hypophysis. The importance of the hypothalamic-hypophyseal stalk in eliciting a thyroid response to cold stress is well known(8).

It was observed in stress experiments that gentling failed to alter the effects of starvation or food intake restriction, provided that animals were given only 1 wk. of gentling before initiation of stress (Table II, Fig. 1). When the pre-stress gentling period was extended to 2 wks., gentling appeared to give starved rats an advantage which was extended over the entire period of stress (Table III, Fig. 2). In Weininger's experiments, animals were gentled for 3 wks. prior to prolonged starvation period. All non-gentled rats succumbed whereas none of the 6 gentled animals died as a result of this experience. Therefore it appears that 2 or more wks. of pre-stress gentling are necessary to influence the animal's ability to withstand the stress of starvation.

In any given experiment, thyroid I-131 uptake values for starved animals were higher than those for non-starved controls (Table III). Animals which were without food when uptake measurements were made, had higher values than those placed back on food. These observations coincide with those of Catz *et al.* (9) who have shown that I-131 uptakes performed after starvation are much higher because the animal received no iodide during this period, and not because of any obvious structural changes in thyroid gland. Therefore thyroid uptake measurement may not be valid for ascertaining the status of thyroid activity in starved animals. However, non-gentled animals in all cases showed higher uptake values than gentled groups, and if these differences are real, non-gentled animals with higher metabolic rates would naturally suffer greatest weight loss under stress of starvation.

When electrical shock was employed, gentled animals lost the growth and food utilization advantage acquired during the first 2 wks. of gentling (Table III, Fig. 3). Shocked animals in both groups had markedly higher uptake values than non-gentled-non-shocked controls, and gentled-shocked animals had higher uptake values than non-gentled-shocked animals. Although these latter differences were not statistically significant, the overall change in thyroid uptake suggests an increase in thyroid function for the shocked groups. This in turn might account for the inferior growth and food utilization performance of these animals.

Summary. Small but statistically significant differences in growth and food utilization were produced in the albino rat through gentling. Gentled animals also showed a trend toward a lowering of thyroid activity. When animals were gentled for 1 wk. preceding stress, no significant differences in growth, food utilization, carcass length or thyroid function could be found between gentled and non-gentled animals. If this initial gentling period was extended to 2 wk., gentled-starved animals extended their growth and food utilization advantage over the non-gentled group, and showed a consistent trend toward lower thyroid function. Animals which were gentled for 2 wks. preceding electrical shock, lost their growth and food utilization advantage over the non-gentled group,

and both groups were markedly inferior to the non-shocked controls. Thyroid uptake measurements indicated that the basal metabolism of electrically shocked rats was increased over that of non-shocked animals, but no statistically significant differences existed between gentled and non-gentled groups.

The authors wish to express their appreciation to Mrs. June Besig for secretarial assistance and to Messrs. Charles Reiner and Allen Ayres for preparation of the figures.

1. Hammett, Frederick S., *Am. J. Physiol.*, 1921, v58, 196.

2. ———, *Endo.*, 1922, v4, 221.

3. Greenman, M. J., and Duhring, F. Louise, *Breeding and Care of the Albino Rat for Research Purposes,* Philadelphia, Wistar Inst. of Anat. and Biol., 1931.

4. Bernstein, Lewis, *Psychol. Bull.*, 1952, v49, 38.

5. Weininger, Otto, *Science,* 1954, v119, 285.

6. ———, *Canad. J. Psychol.*, 1953, v7, 111.

7. Ruegamer, W. R., Bernstein, Lewis, and Benjamin, John D., *Science,* 1954, v120, 184.

8. Uotila, U. U., PROC. SOC. EXP. BIOL. AND MED., 1939, v41, 106.

9. Catz, Borris, El Rawl, Ihsan, and Geiger, Ernest, *Am. J. Physiol.*, 1953, v172, 291.

Received March 29, 1956. P.S.E.B.M., 1956, v92.

Circulating Rat Cells in Lethally Irradiated Mice Protected with Rat Bone Marrow.* (22421)

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Murphy(1) established that (a) embryos lacking ability to resist growth of heterologous tissue could be rendered as refractory as adults by introduction of an isologous adult spleen or bone marrow and (b) adults, capable of resisting growth of heterologous tissue, could be rendered as receptive as embryos by pretreatment with sublethal total-body X irradiation. Rapidly growing tumor tissues were used as donor material. Over 30 years later, Owen(2) demonstrated circulating erythrocytes genetically and phenotypically distinct from the animal's own, which arose from embryonic erythrocytopoietic transplants of the co-twin through placental anastomosis. Successful homologous and heterologous tissue and protein acceptance by embryos and X-irradiated adults followed by a prolonged or even lasting period of tolerance to these agents has been reported(3-8). Lindsley *et al.*(9) demonstrated erythrocyte mosaicism in sublethally X-irradiated rats that were subsequently injected with homologous rat bone marrow.

* Work performed under USAEC contract No. W-7405-Eng-26.

Previous work(10) in this laboratory showed that C_3H x $101F_1$ mice receiving 950 r of total-body X radiation protected against radiation death by a single intravenous injection of an isologous bone marrow suspension responded relatively well to either rat or sheep RBC antigens 30 days later. However, lethally X-irradiated mice protected by an injection of heterologous bone marrow (Sprague-Dawley rats) failed to respond to rat RBC antigen and responded only very weakly to sheep RBC antigen 30 days later. The establishment of the degree of transplantation of rat hematopoietic tissue became necessarily important in an attempt to interpret the immune response of these heterologously protected mice.

Materials and methods. Normal 12-week-old C_3H x $101F_1$ mice, equally divided in sex, received 950 r and were subsequently protected with heterologous bone marrow suspension. Three types of control mice were run simultaneously: (a) nonirradiated normal, (b) irradiated (950 r) and protected with isologous bone marrow suspension, and (c) nonirradiated but injected with heterologous bone marrow suspension. The irradia-

tion conditions were: 250 kvp, 30 ma, 3 mm Al filter, at 80 cm, ~ 100 r/min. The bone marrow suspension, made to 1 ml in Tyrode solution, was injected intravenously within 4 hours after X irradiation. Heterologous rat bone marrow suspension was obtained from 1 femur and 1 humerus, and the isologous bone marrow from 1 femur. Thirty-four mice were used. The 6 controls and 8 heterologously protected mice were individually caged, body weights recorded, and blood samples from the tail vein taken at various intervals. The remaining heterologously protected mice were killed at intervals for serial study. The quantitative cell count method for an assay of isohemagglutinin reported by Wilkie and Becker(11) was modified to determine the RBC population of blood samples. Pooled mouse anti-rat RBC serum (titer, 128-256) and pooled rat anti-mouse RBC serum (titer, 512-1024) were heated at 56°C for 30 minutes. Blood samples (3-4 drops) taken from the tail vein of mice were collected in a solution containing a 1:3 mixture of 0.1 M versene (pH 6-7) and 0.85% NaCl. After washing one volume of packed cells 3 times with 200-300 volumes of saline, a 1-2% cell suspension was prepared in 0.85% NaCl. To four 10 x 75 mm test tubes, 1 volume (0.1 ml) of the well-mixed cell suspension was added; and (a) 1 volume of normal pooled mouse serum (tube A), (b) pooled mouse anti-rat RBC serum (tube B), (c) pooled rat anti-mouse RBC serum (tube C), and (d) a 1:1 mixture of the 2 antisera (tube D) was added to each of these tubes respectively. The tubes were capped with parafilm, shaken, incubated at room temperature for 10 minutes, shaken, centrifuged at 1000 r.p.m. for 1 minute, and the degree of agglutination determined by gently shaking the RBC "pellet" from the bottom of the test tubes. The tubes were then placed in a Kahn shaker set at 116 oscillations per minute for 20-30 minutes. The free RBC count was made of an aliquot from each tube with Hayem's solution as the diluent. It was decided to use both anti-rat and anti-mouse RBC sera to check the results of one against the other. Furthermore, use of both antisera should permit detection of the presence of possible "sensitized RBC" analogous to the "sensitized" Rh-positive cells, provided that the normal range of free cells in tube D is known. In theory, there should be no free cells in tube D, but in practice 5% may become free under the experimental condition, owing to the weak binding of cell antibodies and the physical shaking of test tubes. The following formulas were employed: (a) % cell recovery $= \frac{B + C - 2D}{A}$ x 100, (b) % rat RBC $= \frac{A + C - B}{2A}$ x 100, and (c) % mouse RBC $= \frac{A - C + B}{2A}$ x 100. The mean recovery of total RBC based on over 90 determinations was 96%. The method of albumin suspension of cells(12), the method of enzyme-treated cells(13), and the anti-gamma globulin method(14) were employed to test for the presence of possible "sensitized" red blood cells.

While this work was in progress, attention was called to the USNRDL report of Nowell *et al.*(15) who showed that, histochemically, rat granulocytes gave positive alkaline phosphatase reaction unlike the mouse granulocytes. They demonstrated by this method the presence of alkaline phosphatase positive cells in lethally X-irradiated mice protected with rat bone marrow suspension. This test was undertaken by employing the method of Rabinovitch and Andreucci(16). A comprehensive report on the double serum-agar diffusion method has been published by Oudin (17), Jennings(18), and Wilson and Pringle (19). Serum samples obtained on the 6th, 13th, 17th, 19th, 27th, 39th, and 72nd days were tested simultaneously by this method against rabbit anti-rat serum with an interfacial titer of 4^9 and against rabbit anti-mouse serum with an interfacial titer of 4^{10}. Mouse and rat sera mixed in proportions of 1:0, 3:1, 2:1, 1:1, 1:2, 1:3, and 0:1 were tested simultaneously against both antisera. These served as positive controls.

Results. None of the individually caged lethally X-irradiated and heterologously protected mice died during the first 30 days. On

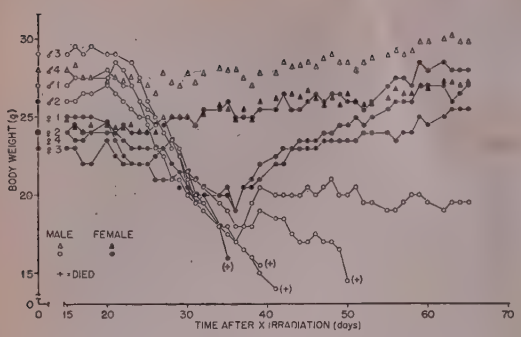

FIG. 1. Effect of rat bone marrow on body wt of lethally X-irradiated (950 r) mice. Control (triangles): avg of nonirradiated mice, irradiated injected with rat bone marrow, and irradiated injected with mouse bone marrow. Experimentals (circles): irradiated mice injected with rat bone marrow.

the 14th day, the heterologously protected mice could not be differentiated from the three types of control mice by gross inspection, and this was also borne out in the distribution of the body weight as shown in Fig. 1. The primary weight loss that occurred about a week after X irradiation was rapidly regained within the following week (heterologously protected males, 26.0-29.0 g; females, 23.0-25 g; control males, 27.0-29.5 g and females, 24.0-25.5 g). Starting on about the 21st day, the heterologously protected mice, except in the case of 1 female, began losing weight precipitously, reaching a minimum on about the 36th day. During this period abundant fecal deposit was observed in

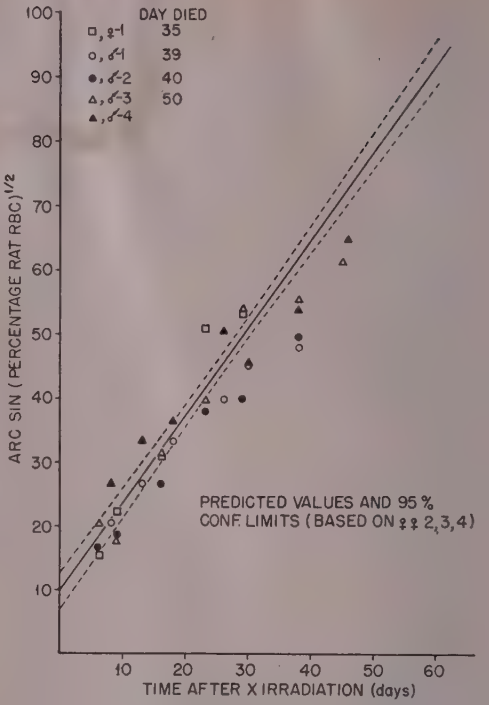

FIG. 3. Rate of percentage rat RBC increase ($y = 10.496 + 1.4027x$).

the cages of these animals. Two of the 4 survivors gradually recovered from the 36th day; one of the 4 showed only partial recovery; the fourth showed no ill effect throughout the 72 days' observation.

The results of the immunohematological study are presented in Fig. 2. The appear-

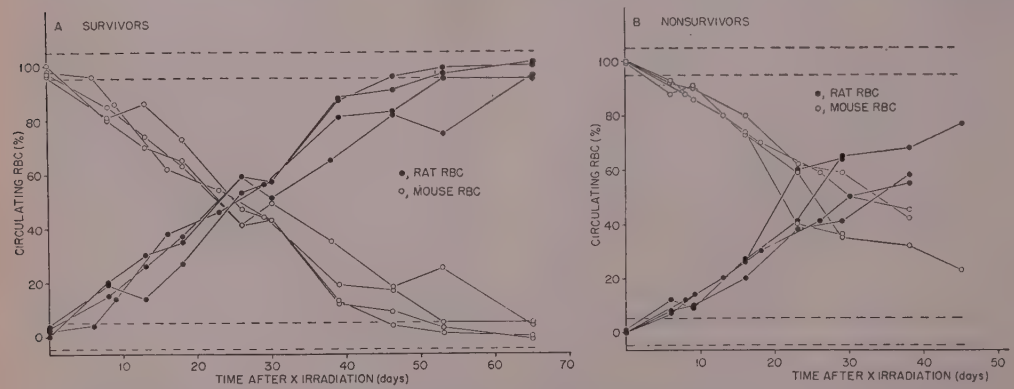

FIG. 2. Appearance of circulating rat RBC in lethally X-irradiated (950 r) mice injected with rat bone marrow. Control: avg of nonirradiated mice, nonirradiated injected with rat bone marrow, and irradiated injected with mouse bone marrow.

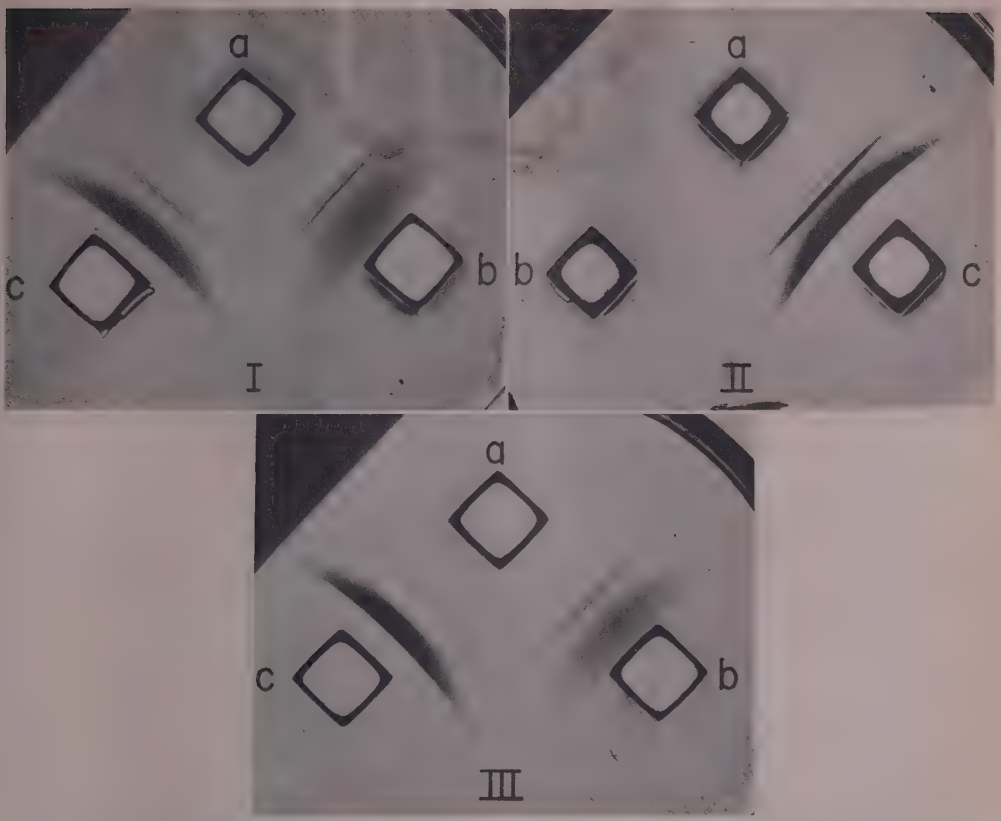

FIG. 4. Serum-agar diffusion pattern after 7 days' incubation. I: a, Normal mouse serum; b, anti-mouse serum; c, anti-rat serum. II: a, Normal rat serum; b, anti-mouse serum; c, anti-rat serum. III: a, Mouse serum 72 days after X-irradiation and rat bone marrow injection; b, anti-mouse serum; c, anti-rat serum.

ance of rat RBC in the circulation became apparent about a week after X irradiation and injection of rat bone marrow. A gradual increase in percentage of rat RBC can be seen in all 8 mice. On about the 25th day, the 50% level was attained. The 95-100% level in rat RBC was approached shortly after the 50th day in the 3 surviving healthy mice, and this level was maintained on the 65th day. With a marked loss in body weight, there was a concomitant decreased rise in percentage of rat RBC. This relation between the body weight change and percentage of rat RBC was evident in comparing the percentage of rat RBC of the 4 mice that died and the surviving male, which did not fully recover, with that of the 3 fully recovered survivors (Fig. 3). No circulating rat RBC could be detected in the control mice throughout the experiment.

Histochemical alkaline phosphatase tests were performed on smears obtained on the 45th and 63rd days. Blood smears from all the heterologously protected mice were positive; those from all the controls were negative. No difference between smears of the normal rats and these heterologously protected mice could be observed. Differential WBC count on smears obtained on the 63rd day showed a relative decrease in the agranulocytic count† from an average of 76% in the controls to 44% in the heterologously protected survivors.

Rat proteins could not be detected by the

† The term "agranulocytic cells" includes lymphocyte, monocyte, and plasmacyte.

double serum-agar diffusion method in serum samples obtained from the 6th to the 72nd days. A typical result is shown in Fig. 4. On the 72nd day, serum samples included the control mice and the 4 surviving heterologously protected mice.

All tests for the presence of "sensitized" RBC were negative. A weakly positive reaction was observed with the trypsin method, but control trypsin tested rat RBC gave a similar result.

Autopsies performed on the 4 mice that died showed no gross evidence of infection or hemorrhage. The most pronounced changes were extreme weight loss and atrophy of lymphatic tissue. The bone marrow in two animals examined microscopically showed about 75% of the normal cellularity in the femoral bone shaft and sternum.

Discussion. It has been shown that approximately 50% of the mice receiving lethal X irradiation and a subsequent intravenous injection of rat bone marrow died in 30 days (10). There were 5-10 mice in each cage. Others(20,21) have obtained similar results since Congdon and Lorenz(22) first demonstrated the protective ability of heterologous bone marrow. Results presented here show that none of these heterologously protected mice died during this period when they were individually caged. In 2 weeks, recovery from the critical X-irradiation effect occurred, as expressed by their gross appearance and body weight, but 1 week later, these mice began losing weight and continued to do so to the 5th and 6th weeks. Associated with the loss in weight was observed an abundant daily firm fecal deposit indicating normal or greater than normal food intake. During this critical period after the 4th week, 3 of 4 males and 1 of 4 females died. A period of general recovery then ensued. Surviving females appeared to be fully recovered by the 8th week and could not be differentiated from the controls. The surviving male, however, did not fully regain its original body weight.

Quantitative immunohematological studies showed that in 8 of 8 mice, transplantation of rat erythrocytopoietic cells took place. A week after X irradiation and a subsequent injection of rat bone marrow, rat RBC first appeared in the circulation and by the 25th day contributed 50% of the circulating RBC. On the 65th day in 4 of 4 surviving mice, 100% rat RBC was present in the circulation, and this level was already approached on the 53rd day in the 3 healthy females.

That rat granulocytopoietic cells also transplanted was shown by the positive alkaline phosphatase test performed on blood smears obtained on the 45th and 63rd days. A similar result was obtained by Nowell *et al.* (15). Differential WBC count on blood smears obtained on the 63rd day showed a relative decrease in the agranulocytic cells. Double serum-agar diffusion tests on serum samples showed no rat serum proteins. The presence of the rat gamma globulin would probably have been indicative of transplantation of plasmacytopoietic cells, which are also found in the bone marrow. It has been demonstrated by many workers that plasmacytic cells are involved in the production of antibody, a modified gamma globulin(23-27).

It can be concluded that injection of rat bone marrow to lethally X-irradiated mice permitted transplantation of erythrocytopoietic and granulocytopoietic cells as expressed in the appearance of the rat RBC and granulocytes in the circulation. The failure of transplantation of rat agranulocytopoietic cells is suggested indirectly by the absence of rat serum globulin and the relative decrease in the agranulocytic count of blood smear. Additional evidence for this concept will depend on the establishment that the host cells, rather than the donor cells, are the antibody producers. The failure to respond to rat RBC antigen by these mice(10) is caused by a complete replacement of the RBC production by the transplanted rat erythrocytopoietic cells. The sequential correlation of (a) loss in weight, (b) death-time relation, (c) immune response to rat and sheep RBC antigens, and (d) the activity of transplanted cells strongly suggests that the delayed death pattern is caused by an *in vivo* antigen-antibody reaction. A report on the delayed death pattern of these mice undertaken by Congdon of this Laboratory will follow(28).

Summary. 1. Mice receiving 950 r can be protected from the 30-day irradiation death by injecting rat bone marrow and caging them individually. 2. Transplantation of rat erythrocytopoietic and granulocytopoietic cells has been demonstrated by the appearance of rat RBC and granulocytes in the circulation. On the 65th day, 100% rat RBC was present in the circulation of all the surviving heterologously protected mice. 3. Rat serum proteins could not be detected in these mice by the serum-agar method.

The author wishes to thank Mr. W. D. Gude and Drs. N. Gengozian, C. C. Congdon, A. W. Kimball, and N. G. Anderson of this Laboratory for their aid and valuable suggestions, which made this work possible.

1. Murphy, J. B., *J. Exp. Med.,* 1914, v19, 513; *J. Am. Med. Assn.,* 1914, v62, 1459

2. Owen, R. D., *Science,* 1945, v102, 400.

3. Clemmensen, J., *The Influence of X-Irradiation on the Development of Immunity to Heterologous Transplantation of Tumors,* Levin and Munksgaard, Ejner Munksgaard, Copenhagen, 1938.

4. Billingham, R. E., Brent, L., and Medawar, P. B., *Nature,* 1953, v172, 603.

5. Main, J. M., and Prehn, R. T., *J. Nat. Cancer Inst.,* 1955, v15, 1023.

6. Hanan, R., and Oyama, J., *J. Immunol.,* 1954, v73, 49.

7. Cinader, B., and Dubert, J. M., *Brit. J. Exp. Path.,* 1955, v36, 515.

8. Dixon, F. J., and Maurer, P. H., *J. Exp. Med.,* 1955, v101, 245.

9. Lindsley, D. L., Odell, T. T., and Tausche, F. G., PROC. SOC. EXP. BIOL. AND MED., 1955, v90, 512.

10. Makinodan, T., and Gengozian, N., unpublished.

11. Wilkie, M. H., and Becker, E. L., *J. Immunol.,* 1955, v74, 192, 199.

12. Diamond, L. K., and Denton, R. L., *J. Lab. Clin. Med.,* 1945, v30, 821.

13. Morton, J. A., and Pickles, M. M., *Nature,* 1947, v159, 779.

14. Coombs, R. R. A., Mourant, A. E., and Race, R. R., *Brit. J. Exp. Path.,* 1945, v26, 255.

15. Nowell, P. C., Cole, L. J., Habermeyer, J. G., and Roan, P. L., USNRDL-TR-59, NM 007015, 2 Sept., 1955.

16. Rabinovitch, M., and Andreucci, D., *Blood,* 1949, v4, 580.

17. Oudin, J., *Methods Med. Res.,* 1952, v5, 335.

18. Jennings, R. K., *J. Bacteriol.,* 1954, v67, 559, 565.

19. Wilson, M. W., and Pringle, B. H., *J. Immunol.,* 1954, v73, 232.

20. Barnes, D. W. H., and Loutit, J. F., *Nucleonics,* 1954, v12(4), 68.

21. Cole, L. J., Habermeyer, J. G., and Bond, V. P., *J. Nat. Cancer Inst.,* 1955, v16, 1.

22. Congdon, C. C., and Lorenz, E., *Am. J. Physiol.,* 1954, v176, 297.

23. Bjorneboe, M., and Gormsen, H., *Acta pathol. et microbiol. Scandinav.,* 1943, v20, 649.

24. Fagraeus, A., *Antibody Production in Relation to the Development of Plasma Cells,* The State Bacteriological Laboratory, Essette aktiebolag 707166, Stockholm, 1948.

25. Coons, A. H., Leduc, E. H., and Connolly, J. M., *Fed. Proc.,* 1953, v12, 439.

26. Makinodan, T., Ruth, R. F., and Wolfe, H. R., *J. Immunol.,* 1954, v72, 45.

27. Good, R. A., *J. Lab. and Clin. Med.,* 1955, v46, 167.

28. Congdon, C. C., unpublished.

Received March 22, 1956. P.S.E.B.M., 1956, v92.

Citric Acid Cycle Enzymes in Normal and Syphilitic Rabbit Tissues.* (22422)

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Numerous attempts at the *in vitro* cultivation of pathogenic *Treponema pallidum* by various investigators have been unsuccessful (Reviewed in Ref. 1). Therefore, it appears advisable to follow other approaches to this problem. *T. pallidum* like a number of other microorganisms shows a preference for specific tissues. Thus, in the rabbit, regardless of the site of inoculation, a syphilitic orchitis develops. A comparative study of the biochemistry of various tissues, both infected and non-infected, might possibly point to factors required for the growth of the organism. The present investigation is concerned with concentrations in tissues of most enzymes of the citric cycle: α-ketoglutaric oxidase (hereafter referred to as KGO), pyruvic oxidase (PO), fumarase (FU), aconitase (AC), triphosphopyridine nucleotide (TPN) isocitric dehydrogenase (ICD) and succinic dehydrogenase (SD) together with lactic dehydrogenase (LD).

Methods. Preparation of rabbits. Nineteen normal animals were used in 11 experiments. In 6 rabbits of this group trauma was produced by injecting into each testis 1 ml of a mixture containing equal parts of 0.85% NaCl solution and normal rabbit serum. These rabbits were killed 7, 14, 20, 29 and 48 days after trauma was produced. Because no difference was found between these two groups of uninfected animals they were all considered as normal rabbits (Table I). There were 29 rabbits in 14 experiments in the syphilitic group. The Nichols strain of *Treponema pallidum* was employed. 10^7 organisms in 1 ml saline-serum suspension were injected into each testis. The syphilitic rabbits were killed at weekly intervals during a period of 8 to 104 days after infection. *Preparation of tissues for assaying.* Immediately after the death of the animals, the organs were removed, trimmed of fat and connective tissue, then frozen and stored at −10°C until used. For assaying 4 enzymes FU, KGO, PO and LD one extract was prepared as follows: 0.5 g portion of each organ was homogenized in a glass homogenizer with 4.5 ml of sucrose-phosphate solution of pH 6.70 (containing 34.24 g sucrose, 100 ml of 0.1 M Na_2HPO_4, 100 ml 0.1 M KH_2PO_4 and distilled water to make a final volume of 400 ml). The homogenates were centrifuged at 6000 g at 4° for 40 minutes in a Servall angle centrifuge. The supernatants were used for assaying these 4 enzymes. A similar procedure was used for AC assaying, but here the extracting solution contained 0.002 M sodium citrate and 0.002 M citric acid(2). For assaying ICD the extracting solution contained 171 g of sucrose, 500 ml of 0.05 M barbital buffer of pH 7.50 and distilled water to make 2000 ml. Here 0.4 g of each tissue was homogenized with 19.6 ml of the sucrose-barbital solution and the homogenate was filtered through 6 layers of cheese cloth. This homogenate was assayed without centrifuging. In those instances where the supernatants of the specially prepared homogenates have been used, the respective enzymes were largely present in the supernatants of the homogenates. *Assaying procedures.* α-Ketoglutaric oxidase and PO were assayed spectrophotometrically using 2,6-dichlorophenol indophenol as the oxidant(3). One ml of sucrose-phosphate solution of pH 6.70 (described in the preceding paragraph), 300 μg (0.1 ml) of cocarboxylase, 0.1 ml or 0.15 ml of the centrifuged (10%) homogenate and 0.3 ml neutralized KCN (0.1 M) were placed in a test tube and mixed. The mixture was allowed to stand for 1 minute at room temperature, in order to inactivate the enzyme which

* Presented before the Am. Soc. for Exp. Pathol. at meeting of Fed. of Am. Soc. Exp. Biol., Atlantic City, N. J., April, 1956.

TABLE I. Citric Acid Cycle Enzymes in Testes (Units/mg Dry Basis).

	KGO	PO	FU	AC	LD	ICD
			Avg of normal group			
	24	22	10	11	80	5
			Activity ranges of normal groups			
	13–34	11–37	7–13	9–16	62–101	3–8
			Avg of syphilitic groups			
(8– 14)*	14	3	11	5	84	4
(21– 40)	7	6	10	5	91	3
(45– 72)	11	10	14	6	91	4
(73–104)	14	15	16	7	73	5
			Avg of 4 syphilitic groups			
	11	9	13	6	84	4
			Activity ranges of syphilitic groups			
	5–21	2–18	7–17	2–9	60–150	2–7

* Figures in parentheses indicate period of infection in days.

reoxidizes the reduced dye; then 0.4 ml of 0.01% dye solution and 60 μM sodium pyruvate or neutralized α-ketoglutaric acid were added per final volume of 3 ml reaction mixture. The reaction mixture was transferred to a cuvette and the decrease in optical density (OD) at 600 mμ was observed. All enzyme activities were measured at 25.5°. One unit is defined as that amount of enzyme which produces a decrease in OD of 0.001/min. Fumarase and AC were assayed at 250 mμ and 240 mμ respectively at pH 7.40 by the method of Racker(4). One unit of AC and FU activity is defined as the quantity of enzyme causing an increase in OD of 0.001/min. ICD was measured by observing the rate of reduction of TPN in the presence of manganous ions and isocitrate at pH 7.50 at 340 mμ(5). One unit is defined as the quantity of enzyme which causes an increase in OD of 0.01/min. LD was assayed by measuring the rate of reduction of DPN at pH 10.00 at 340 mμ(6). One unit of enzyme activity is defined as the quantity of enzyme causing an increase in OD of 0.001/min. SD assays(7) were done on whole homogenates (20%) measuring tetrazolium reduction at pH 7.40 in 1 hour at 37° in the Klett-Summerson colorimeter. A calibration curve was obtained by reduction of the tetrazolium salt with 0.3 ml of a 10% solution of sodium hydrosulfite per tube (25 to 200 μg triphenyltetrazolium chloride). This method produced maximum and stable color. One unit of enzyme activity is defined as μg of tetrazolium salt reduced per mg of tissue under the condi-

TABLE II. Citric Acid Cycle Enzymes in Liver (Units/mg Dry Basis).

	KGO	PO	FU	AC	LD	ICD	SD
			Avg of normal group				
	18	19	56	14	124	17	2
			Activity ranges of normal groups				
	8–28	8–32	42–70	12–16	72–189	9–28	1–3
			Avg of syphilitic groups				
(8– 14)*	18	16	37	9	89	16	2
(21– 40)	16	12	50	13	109	16	2
(45– 72)	20	18	52	10	147	15	1
(73–104)	22	21	58	9	141	13	1
			Avg of 4 syphilitic groups				
	19	17	51	10	127	15	1
			Activity ranges of syphilitic groups				
	8–33	8–28	34–74	5–15	72–203	11–18	0-3

* Figures in parentheses indicate period of infection in days.

TABLE III. Citric Acid Cycle Enzymes in Heart (Units/mg Dry Basis).

	KGO	PO	FU	LD	ICD	SD
			Avg of normal group			
	12	8	192	725	18	1
			Activity ranges of normal groups			
	7–16	3–11	86–248	426–881	12–27	0–3
			Avg of syphilitic groups			
(8– 14)*	22	8	177	677	27	1
(21– 40)	17	5	218	696	17	2
(45– 72)	7	5	224	682	22	1
(73–104)	9	5	218	652	23	2
			Avg of 4 syphilitic groups			
	12	6	214	677	21	1
			Activity ranges of syphilitic groups			
	5–37	3–11	154–246	518–834	4–34	1–3

* Figures in parentheses indicate period of infection in days.

tions of the assay.

Results. Testes. The results given in Table I demonstrate that during the most virulent stage of infection (8 to 14 days) PO dropped to 1/8th of the normal range and later (after 21 to 40 days of infection) the values were 1/4th those of the normal range. A less pronounced but qute significant lowering in KGO, AC and ICD activities took place. Both normal and syphilitic testes contained only a trace of SD. The FU activity range was the same in normal and syphilitic testes. *Liver.* In the livers of two infected rabbits (72 and 104 days after infection) SD was completely absent. The other enzymes of this organ were within the normal range of activity (Table II). *Heart.* Both normal and syphilitic heart tissue showed considerable variations in the ranges of AC activity (2 to 62 units for normal tissue and 2 to 119 units for syphilitic tissue), and because of this the aconitase figures have been omitted from Table III. *Kidneys.* There was a gradual decrease of PO (within a wide range) in syphilitic kidneys. There was a moderate decrease in AC activity. The other enzyme activities of kidneys varied within a wide range so that no significant differences could be observed between normal and infected tissues (Table IV).

DPN isocitric dehydrogenase was not determined since the enzyme was found to be present only in traces in rabbit tissues. It may be seen that of the normal rabbit tissues studied testes contained most KGO and PO, the heart contained most FU and LD, and

TABLE IV. Citric Acid Cycle Enzymes in Kidneys (Units/mg Dry Basis).

	KGO	PO	FU	AC	LD	ICD	SD
			Avg of normal group				
	23	24	173	55	213	23	2
			Activity ranges of normal groups				
	16–31	16–34	125–194	48–62	178–264	18–32	1–4
			Avg of syphilitic groups				
(8– 14)*	31	17	178	43	176	17	1
(21– 40)	26	11	133	40	187	21	1
(45– 72)	15	8	155	35	190	23	2
(73–104)	15	13	146	47	181	23	2
			Avg of 4 syphilitic groups				
	20	12	150	41	185	23	2
			Activity ranges of syphilitic groups				
	8–46	4–28	114–218	22–62	106–247	15–31	1–3

* Figures in parentheses indicate period of infection in days.

kidneys contained most AC, ICD, and SD. These results are reported on dry basis. They appear to be just as significant when evaluated on wet basis.

Summary. 1. Normal and syphilitic rabbit testes, liver, heart and kidneys were assayed for α-ketoglutaric oxidase, pyruvic oxidase, fumarase, aconitase, lactic dehydrogenase, TPN isocitric dehydrogenase and succinic dehydrogenase. 2. In syphilitic testes there is a definite decrease in pyruvic oxidase, α-ketoglutaric oxidase, aconitase and TPN isocitric acid dehydrogenase. 3. Both normal and syphilitic heart tissue showed very considerable variations in aconitase activity. 4. In syphilitic kidneys there was a decrease in pyruvic oxidase activity and a moderate decrease in aconitase activity. 5. The decreased enzyme values found in the testes and kidneys of the syphilitic groups are probably a demonstration of a general reaction to the infection.

The author appreciates the assistance of John W. Clark, Jr., Carl S. Adams, and David L. Johnson.

1. Saurino, V. R., and DeLamater, E. D., *Am. J. Syph., Gonor. and Ven. Dis.,* 1952, v36, 353.

2. Anfinsen, C. B., *Methods in Enzymology,* 1955, v1, 695.

3. Sanadi, D. R., and Littlefield, J., *J. Biol. Chem.,* 1952, v197, 851.

4. Racker, E., *Biochim. et biophys. acta,* 1950, v4, 211.

5. Ochoa, S., *Methods in Enzymology,* 1955, v1, 699.

6. Neilands, J. B., *ibid.,* 1955, v1, 449.

7. Kun, E., and Aboad, L. G., *Science,* 1949, v109, 144.

Received April 3, 1956. P.S.E.B.M., 1956, v92.

Comparative Biochemical Studies on Normal and Poliomyelitis Infected Tissue Cultures. IV. Enzyme-changes in Host Cells. (22423)

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The successful cultivation of the virus of poliomyelitis by Enders *et al.*(1) provides a unique opportunity to approach host-virus interaction in simple systems by excluding inflammatory reactions. Enzymes connected with nucleic acid metabolism were extensively studied in explants of normal Rhesus kidney with synthetic nutrients and in surviving tissues(2-4) to assure a baseline for the present work. Alterations occurring in similar biocatalysts during *in vitro* growth of poliomyelitis virus are here reported. Since enzymology in normal and in infected tissue culture (TC) is a relatively unexplored field(2,3) a brief synopsis of findings on nine different enzyme systems is presented. Details, together with various secondary problems investigated, will be published separately(5).

Methods. Assay material was obtained from the same source† as previously. In preliminary publications preparation of TC, media and homogenates was described, together with the assay methods for enzymes(2,3). Adherence to these technics makes repetition unnecessary and assures continuity with previous communications. The trypsinized Rhesus kidney epithelial cells were routine material from virus titrations, grown in roller tubes with synthetic medium 199(6) or its modified form, 597(2). All virus work was carried out in the poliomyelitis department. Titres were expressed in negative logarithms calculated by Kärber's method(7). Mahoney, MEFI and Saukett virus types were used. The strains were from TC passages and generally of high dilutions ($10^{-6.5}$ to $10^{-7.5}$). Sin-

* Aided by a Public Health Research Grant.

† Through the courtesy of Drs. A. E. Franklin, D. R. E. MacLeod, and H. G. Macmorine of poliomyelitis department of Connaught Medical Research Laboratories.

TABLE I. Turbidity Readings of Infected TC.

Explants,* nutrients, type of virus†	Virus titer	Turbidity, Klett units/ml
Roller tube cultures of trypsinized kidney cortex:		
Normal TC in medium 199, homogenates, pool of 50 tubes	—	900
Same, infected with Mahoney strain	$10^{-6.7}$	670
Idem	10^{-7}	610
Normal TC fluid in medium 597, pool of 20 tubes	—	30
Same, infected with Mahoney strain	$10^{-7.5}$	25
Normal TC extract in medium 597, pool of 20 tubes	—	45
Same, infected with Mahoney strain	$10^{-6.8}$	35

Total (homogenates)	10 exp.	Normal (mean)	575
		Infected "	515
Total (TC fluids)	4 "	Normal "	27
		Infected "	21

* 14 days' TC, except second pair, which were 9 days'.
† 6 days' infection, incubation at 37°C, except of second pair, which was of 1 day infection.

gle roller tube cultures assayed for enzymes individually gave a maximum variation of about ± 10%. Thus pools were prepared from as many tubes as possible, to reduce sampling error. When supernatants of TC fluids were employed they were mixed without shaking and centrifuged at 3000 r.p.m. for 10 minutes. The work with homogenates necessitated grinding of cells in the original tubes as reported(3). Tissue cultures were used fresh, usually after 6 days incubation with virus, occasionally stored at 4°C, or frozen at –25°C. Turbidity measurements of the pools were carried out by Loomey's technic as a check on tissue mass of homogenates and TC fluids. U.V. spectrophotometry was also used for this purpose, carried out on supernatants(2). The methods of Shinowara *et al.*(2,3,9) served for detection of acid and alkaline phosphatases (Na-glycerophosphate substrate), combined with Fiske and Subbarow's technic(10) for inorganic phosphorus (IP). Reis' method(11) for 5-nucleotidase with muscle adenylic acid and similar technics for simple nucleotidases with mixed ribonucleotides as substrate are adopted(2,12, 13). The total acid soluble phosphorus method for RNA-ses(2,3,14) and McCarty's(15) viscosimetric technics for DNA-se, as modified by Siebert *et al.*(16) were used, with highly polymerized nucleic acids as substrates (2-4). For pseudo-cholinesterase determination a spectrophotometric method was used (2,17), with benzoylcholine substrate. All experiments were on different batches and the assays were carried out at least in duplicate. Mean values of fully randomized samples are tabulated.

Results. A great number of infected TC were assayed, always controlled with identically treated, but uninoculated normals. Results are presented in six tables and two figures. Table I illustrates decrease in turbidity measurements of TC homogenates at the height of virus growth. The difference in mean values of random samples from infected and uninfected cultures is considerable. There seems to be linearity between virus titres and decrease in turbidity. Typical examples of supernatant fluid and centrifuged homogenates illustrate the small amount of material present and the difference between normal and infected samples with respect to turbidity. This is further evidenced by a characteristic ultraviolet absorption curve, which demonstrates qualitative and quantitative change in the medium due to the presence of virus(Fig. 1).

Table II presents examples and a summary of results for acid and alkaline phosphatase assays. The general pattern was a sharp decrease in both enzyme activities in infected cells. The difference between randomized normal and infected pools was pronounced in fresh tubes. However, if the cultures are stored for various times, after 6 days infection and incubation with virus, the pattern may

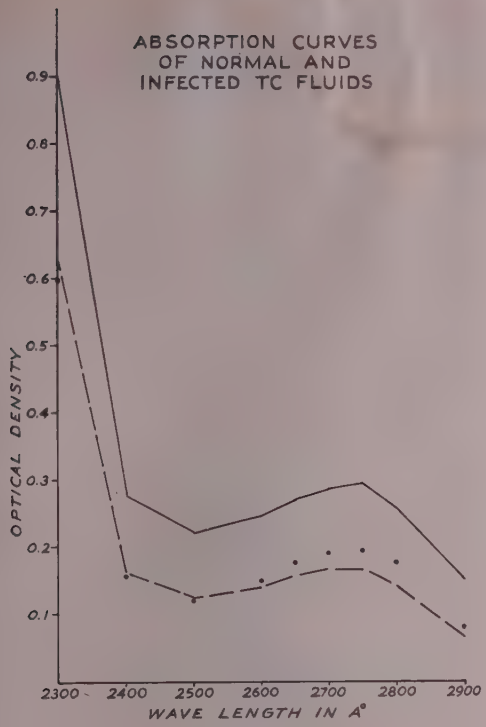

FIG. 1. ——— Normal, 1 ml, in phosphate buffer, pH 7.4. — — — Same, after 3 days infection with Mahoney strain, titer 7.3, dilution 10^{-5}. ● Fresh medium 597, 1 ml, in same buffer. Reference: phosphate buffer.

be modified(5). By calculation of mean values in this synopsis, composition of nutrients, age of TC, virus strains, titers, and fractions of TC were disregarded.

Tables III and IV illustrate the behaviour of 5-nucleotidase and simple nucleotidases. The former (Table III) is less sensitive to the effect of virus than the latter, or than the phosphatases. They may enter the supernatant mostly during cell disintegration(2,3), persist there much longer and suffer less decrease, or even show an increase in some old cultures with low virus titers(5). Simple or nonspecific nucleotidases (Table IV) are very sensitive to virus and their decrease is connected in part with the decrease of phospha-

TABLE III. 5-Nucleotidases in TC Infected with Poliomyelitis Viruses.

Explants,* nutrients, type of virus†	Virus titer	5-Nucleotidase, as increase in IP (μg/ml), pH 8.5, incubation 2 hr, 37°C
Roller tube culture of trypsinized kidney cortex:		
Normal TC in medium 199, homogenates, pool	—	1.53
Same, infected with Mahoney strain	10^{-7}	.64
Total: 20 exp. (TC fluids and homogenates)	Normal (mean)	.88
	Infected "	.58

* 14 days' TC.
† 6 days' infection and incubation at 37°C.

TABLE IV. Simple-Nucleotidases in TC Infected with Poliomyelitis Viruses.

Explants,* nutrients, type of virus†	Virus titer	Simple-nucleotidase as increase in IP (μg/ml), pH 8.5, incubation 2 hr, 37°C
Roller tube cultures of trypsinized kidney cortex:		
Normal TC in medium 199, supernatant, pool	—	1.92
Same, infected with Mahoney strain	$10^{-6.5}$	.73
Total: 10 exp. (TC fluids and homogenates)	Normal (mean)	.81
	Infected "	.49

* 14 days' TC.
† 6 days' infection and incubation at 37°C.

TABLE II. Phosphatase in TC Infected with Poliomyelitis Viruses.

Explants,* nutrients, type of virus†	Virus titer	Acid and alkaline phosphatase increase in IP (μg/ml), incubation 4 hr at 37°C	
		pH 5	pH 10.9
Roller tube tissue cultures of trypsinized kidney cortex:			
Normal TC in medium 597, supernatant, pool	—	.64	1.73
Same, infected with Mahoney strain	$10^{-6.5}$	.16	.29
Total: 20 exp. (TC fluids and homogenates) Normal (mean)		.46	.87
Infected "		.28	.37

* 14 days' TC. † Example is of 5 days' infection.

TABLE V. Effect of Poliomyelitis Viruses of Ribonucleases of TC.

Explants,* nutrients, type of virus†	Virus titer	RNA-se, as increase in total acid soluble phosphorus (μg/ml) pH 5	pH 7.6
Roller tube culture of trypsinized kidney cortex:		Incubation 4 hr, 37°C	
Normal TC in medium 597, homogenate, pool	—	8.80	0
Same, infected with Mahoney strain	$10^{-7.1}$	4.00	0
		Incubation 2 hr, 37°C	
Normal TC in medium 597, supernatant, pool	—	1.86	2.98
Same, infected with Saukett strain	$10^{-6.9}$	2.26	2.40
Normal, whole TC in medium 597, original tubes	—	2.80	2.06
Same, infected with MEF strain	$10^{-6.8}$	4.00	5.26
Total: 15 exp. (TC fluids and homogenates) Normal (mean)		15.40	5.80
Infected "		6.96	9.80

* 14 days' TC. † 6 days' infection and incubation at 37°C.

tases(2,3).

Two types of ribonucleases, an acid and an alkaline, were examined in the infected TC (Table V). The examples presented show a drop of RNA-se activity in the infected TC at the time of "harvest" although exceptions are also illustrated individually and in mean values as an increase of RNA-se activity. The large individual variation of nucleases in normal(2,3) and infected samples makes evaluation of the findings somewhat complicated. However, much significance should be attached to the increase of RNA-ses in the early phase of the infection, which seems to be connected with the synthesis of the virus(5). Table VI presents findings on two types of DNA-se. Both main groups exhibit a marked decrease in nuclease activity at the height of poliomyelitis infection. Similar behaviour of the pseudo-cholinesterase(2) was observed in preliminary experiments. It showed no activity in infected TC fluid (Fig. 2) as measured by decrease of optical density at 2400 Å. Spectra of TC and substrate alone, in buffer did not show any change during the same period of incubation.

TABLE VI. DNA-ses in TC Infected with Poliomyelitis Viruses.

Explants,* nutrients, type of virus†	Virus titer	DNA-se activity, as % drop of relative viscosity, incubation 2 hr, 37°C pH 5	pH 7
Roller tube culture of trypsinized kidney cortex:			
Normal TC in medium 597	—	7.0	5.4
Same infected with Mahoney strain	$10^{-6.5}$	3.0	0
Total: 15 exp. Normal (mean)		9.0	2.0
(TC fluids and homogenates) Infected "		3.0	1.0

* 14 days' TC.
† 6 days' infection and incubation at 37°C.

Discussion. It was thought that explanta-

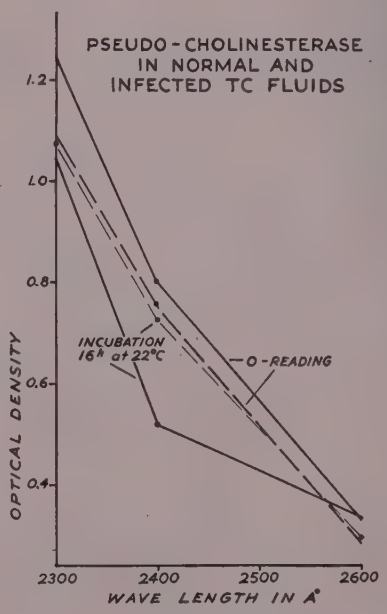

FIG. 2. ——— Normal, 0.25 ml + benzoylcholine (5×10^{-5} mol.) substrate, in phosphate buffer 7.4. — — — Same, infected with Mahoney strain, titer 6.5, dilution 10^{-7}. Reference: phosphate buffer.

tion and cultivation procedures render non-neural tissue susceptible to poliomyelitis infection(2,3). The present findings shed light on the general effect of the virus upon the host cell. At the peak of the growth curve there is a considerable drop in the enzyme activities of fresh TC with complete synthetic nutrients. This change precedes the cytopathogenic effect by at least 24 to 48 hours, depending on virus titers, and can be measured quantitatively. Some enzymes go through a maximum before decrease. Day-to-day experiments on portions of large batches revealed the various phases of these changes(5) due to the presence and later to the multiplication of virus. It remains to be decided if the enzymes are destroyed or only temporarily inhibited. The decrease of their activity may be caused by the virus directly, or by some toxic substances elicited by its presence, because this deleterious effect can be transferred with infected TC fluids in the presence of crude or purified enzyme systems *in vitro*(8). This indicates that a strong inhibitory mechanism must be present, which, like the virus, is carried through the serial dilutions. Various enzymes were sensitive to varying degrees to the action of virus. However, this fact does not protect the cell from the disorganization of its functional balance, especially if essential biocatalysts have been attacked. This results in severe alterations of the metabolism, with progressive accumulation of metabolites and virus, leading to (necrosis) death and disintegration of the cells. The acid RNA-se and 5-nucleotidase seem to be the most resistant, while acid phosphatase, simple-nucleotidases, pseudocholinesterase and alkaline phosphatase are the most sensitive to virus effect. Because of the very large amount of material these biocatalysts will be described individually in detail(5). In this way many important problems connected with the subject can be dealt with. Finally, decrease in turbidity and in optical density of infected TC may be due to disturbed nucleoprotein metabolism and suggests that basic cell constituents may have been destroyed and not replaced during one or another phase of the invasion. It is striking how clearly the absorption curve reflects the modified metabolism and interchange of substances between medium and cells. It remains to be settled whether the point of attack of the virus is primarily at the site of nucleolytic enzymes, or whether damage of oxidative biocatalysts(9,10) represents the first stage in this profound alteration of cell physiology. Further studies are being made to correlate the nuclease pattern observed in the CSF of poliomyelitis patients(13) with the findings presented.

Summary. 1. Marked changes in the activities of 9 enzyme systems were observed at the height of poliomyelitis infection *in vitro*. 2. The decrease of alkaline phosphatase, pentanucleotidase, simple nucleotidase, "acid" RNA-se and DNA-se is so marked, that this may serve as a quantitative measure of cytopathogenic effect of the poliomyelitis virus. 3. The bearing of these findings on cellular physiology and pathology is discussed, as a working hypothesis.

The technical help of Mr. Louis Szendy and Mr. Joseph Kovacs is gratefully acknowledged.

1. Enders, J. F., Weller, T. H., and Robbins, F. G., *Science,* 1949, v109, 85.

2. Kovacs, E., *Can. J. Biochem. Physiol.,* 1956, v34, 273.

3. ———, in press, 1956, v34, May issue.

4. ———, *idem.*

5. ———, to be published.

6. Morgan, J. F., Morton, H. J., and Parker, R. C., PROC. SOC. EXP. BIOL. AND MED., 1950, v73, 1.

7. World Health Organization Technical Report Series, 1954, No. 81.

8. Loomey, J. M., and Walsh, A. I., *J. Biol. Chem.,* 1939, v130, 635.

9. Shinowara, G. Y., Jones, L. M., and Reinhardt, L., *ibid.,* 1942, v142, 921.

10. Fiske, C. H., and Subbarow, Y. J., *ibid.,* 1925, v66, 375.

11. Reis, J., *Enzymologia,* 1938-39, v5, 251.

12. Stern, H., Allfrey, W., Mirsky, A. E., and Saetrens, H., *J. Gen. Physiol.,* 1952, v25, 539.

13. Kovacs, E., *J. Pediatrics,* 1955, v46, 691.

14. Kunitz, M., *J. Gen. Physiol.,* 1940-41, v24, 15.

15. McCarty, M., *ibid.,* 1946, v29, 123.

16. Siebert, G., Lang, K., Lucius-Lang, S., Herkert, L., Stark, G., Rossmüller, G., and Jöckel, H., *Hoppe-Seyler's Z. Physiol. Chem.,* 1953, v295, 229.

17. Kalow, W., *J. Pharmacol. Exp. Therap.*, 1952, v34, 122.
18. Kovacs, E., *Proc. Can. Physiol. Soc. 19th Annual Meeting*, London, Ont., 1955, Oct. 22-23.
19. Howe, H. A., and Flexner, J. B., *J. Biol. Chem.*, 1947, v167, 663.
20. Ackermann, W. W., and Johnson, R. R., *J. Exp. Med.*, 1953, v97, 315.

Received April 9, 1956. P.S.E.B.M., 1956, v92.

Transcapillary Loss, Equilibrium Time, Half-Return Time of Thiocyanate and Heavy Water in the Forearm.* (22424)

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We previously described a method for estimating the transcapillary exchange of permeable substances in the human forearm(1). The method later was extended to the pulmonary capillaries of man(2). No attempt was made in these experiments to estimate the length of time the permeable substances remain in the tissues or their rate of return to the circulation. More recently we have described a method for determining the late washout slopes of injected tracer materials in the human forearm(3). The method takes advantage of the fact that the forearm circulation is small in comparison to the general circulation. Thus, brachial arterial injection of tracer materials in dosages sufficient to produce significant concentrations in the effluent ipsilateral veins produces negligible concentrations when diluted in the general circulation. In this way contamination and consequent distortion of the late washout slopes due to recirculation of significant amounts of labelled material can be avoided.

The present report is concerned with the transcapillary loss and later return of the extracellular electrolyte, thiocyanate, and of heavy water. We believe that these studies provide a more complete picture of the circulation of extravascular substances under physiological circumstances than has been available heretofore.

* Supported in part by research grants from the N.H.I., National Institutes of Health, U. S. Public Health Service and the Squibb Institute for Medical Research, New Brunswick, N. J.

Method. Preparation of labelled materials and manner of injection and sampling have been described in other communications(4,1, 3). The method of analysis of thiocyanate and deuterium oxide also has been described previously(1). The subjects were young or early middle-aged males who were on the wards of the Veterans Administration and Georgetown University Hospitals. They all were ambulatory and afebrile at the time of testing and were not suffering from circulatory disease which might interfere with normal transcapillary exchanges in the forearm. Twenty-two tests were carried out using T-1824 and thiocyanate. In 8 subjects D_2O was determined. In 13 tests forearm and hand capillary beds were included. In one of these subjects (M.W., Table I) simultaneous sampling was carried out from cephalic and median veins. In 8 subjects tracer materials were limited to the forearm by inflating a cuff about the wrist to pressures of 100 mm Hg above systolic pressure. In the remaining 2 cases, permeability characteristics of the hand alone were studied, injection being made into a radial artery with subsequent sampling from a vein near the wrist. The site of transcapillary exchange (forearm or hand or both) did not appear to make any significant difference in the results obtained.

Definition of Terms. Methods for determining rate of net return have not been described previously and, therefore, requires some explanation. The method of determining transcapillary percentage loss has been

described previously(1) so that its principles only will be briefly outlined here. Mere injection of a permeable substance with later sampling is not adequate because it is impossible to determine the extent of its dilution by the blood. The present technic combines the permeable tracer materials with an impermeable substance (T-1824). A portion of this mixture is injected into the brachial artery and another portion saved for analysis of the relative concentrations of the various substances in the injectate. 1. *Expected concentration* of each permeable tracer is the concentration of the substance in each sample that would be expected if there were no transcapillary gain or loss as the material passed through forearm circulation. The permeable labelled substance is mixed completely with the impermeable tracer prior to injection. It is assumed that the two travel together and are equally diluted by the circulating blood subsequent to injection into the brachial artery. It does not matter if the pattern of blood flow distribution is uneven in the forearm so long as the impermeable and permeable tracer are distributed to each vascular bed in the same proportionate concentration as was present in the injectate. The plasma samples collected at intervals of 2 to 4 seconds from an effluent vein are analyzed for their concentration of the impermeable tracer (T-1824). Concentrations of both permeable and impermeable substances also are determined in an aliquot of the injected mixture. The expected concentrations of the permeable substance in each sample are calculated as follows: $C^t_x = \frac{C_x}{C_e} \times C^e{}_m$ where C^t_x is the expected concentration of the permeable substance in each sample, C_x the concentration of this substance in the injectate, C_e the injectate concentration of the impermeable tracer and $C^e{}_m$ the respective sample concentration of the impermeable material. Suitable correction must be made for red cell penetration as has been described in a previous report(1). The venous samples also are analyzed for their *actual* concentrations of each permeable tracer. The values of expected and actual concentrations then are plotted on semi-log paper as indicated in Fig. 1.

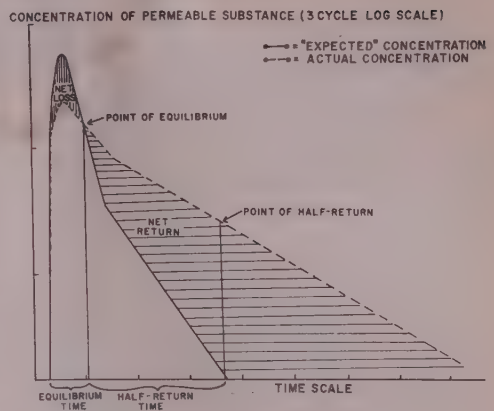

FIG. 1. Graph of "expected" and actual concentrations of a hypothetical permeable labelled substance illustrating the terminology employed. See "Definition of Terms" section in the text for details.

2. *Equilibrium Time.* It will be seen from Fig. 1 that values of actual concentration at first lie below and later cross over to remain above the curve of expected concentrations. The difference between the expected and actual concentrations to the left or prior to the point of crossing represents net losses from blood to the extravascular tissues whereas the difference between the actual and expected concentrations to the right of the crossing indicates net return from the tissues to the blood. The point of crossing therefore represents an equilibrium and the time from appearance to the point of crossing is called the *Equilibrium Time* of the respective permeable tracer. It also represents the total period of net transcapillary loss. 3. *Half-Return Time.* The area enclosed by the respective curves of expected and actual concentrations between the appearance and the point of equilibrium is proportional to the total net loss in the vascular bed drained by the effluent vein used for sampling. The latter is calculated by integrating the net losses per unit time from appearance to equilibrium time (expected minus actual concentrations per unit time). The net returns per unit time (actual minus expected concentrations beginning at the equilibrium point) then are added together until the sum equals half the total net loss. Since

net loss begins at the equilibrium point the time of half return minus the time of the equilibrium point is designated as the *half-return time*. It represents the duration of time in seconds from the beginning of net return to completion of half-return. Since there is no significant recirculation of the tracer materials it is possible to estimate the return to the blood stream of the substances which have permeated into the tissues. Following the passage of the main bolus of injectate through the capillaries of the forearm the subsequent blood entering this vascular bed contains negligible quantities of the permeable tracer materials. This produces a favorable gradient across the capillary wall for determining the rate of return of the permeable tracer substances to the circulation.

Results. Early per cent loss of thiocyanate and deuterium oxide. During the early portion of the transit curves of the labelled materials up to and including the peak values the concentrations in the blood are continuously rising and exceed those in the tissues. Thus, during this period there is a gradient between blood and tissues favoring outward migration of the permeable substances and opposing their inward return. The percentage loss of the SCN and D_2O during this early period, therefore, probably represents a fair approximation of the permeability of the capillary wall to these substances. The percentage losses of SCN and D_2O were determined by dividing the difference between the expected and actual concentrations by the expected concentrations(1). The results listed in Table I represent the percentage losses at the peak. For thiocyanate the mean transcapillary loss was 49 $\pm$ 19%. For deuterium oxide the mean loss was 90 $\pm$ 4.1%. These values are not materially different from those previously reported in a smaller series (1).

Equilibrium time of thiocyanate. The upslope and early downslope of the SCN concentration curve paralleled the T-1824 curve. However, after the early downslope SCN deviated to produce a washout slope with a smaller gradient than that of T-1824. When the expected concentrations of thiocyanate

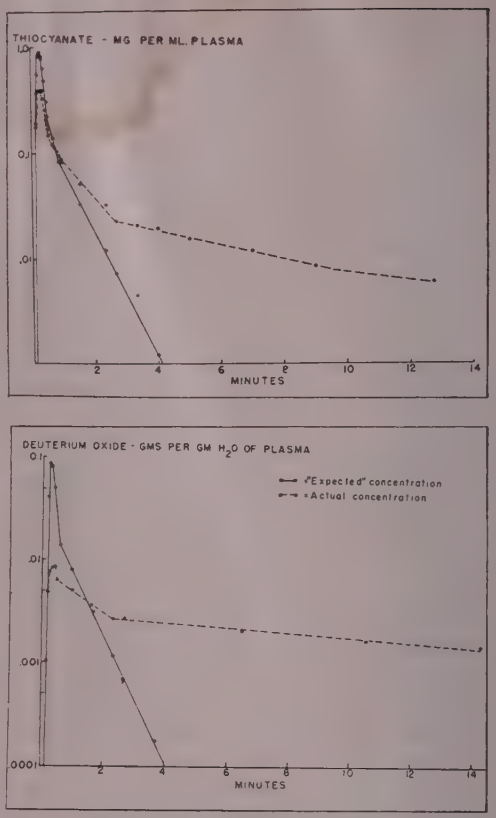

FIG. 2. (Upper graph.) The curves of expected (solid line) and actual (broken line) concentrations are plotted against time. Simultaneously determined curves for deuterium oxide are plotted in lower graph. 19th line of Table I.

were plotted against the actual concentrations the curves crossed at a point in their downslopes as shown in Fig. 2a. This crossing represents the time at which the net loss of thiocyanate equals the net gain (see under "definition of terms," above). The equilibrium time or period of net loss for SCN in the 22 subjects averaged 111 $\pm$ 66 seconds (Table I). The equilibrium time for SCN varied directly with the mean circulation time (T) of T-1824. When the equilibrium times of SCN were plotted against the respective mean circulation times of T-1824 the points were grouped along the line shown in Fig. 3a. The formula for this line indicated that the equilibrium time of SCN was approximately equal to 3 times the mean circulation time of

TABLE I. Per Cent Losses, Equilibrium Times and Half-Return Times for Thiocyanate and Deuterium Oxide in 22 Subjects.

		Thiocyanate				Deuterium oxide				
Age	T_{T-1824},§ sec.	% loss at peak	E.T.,* sec.	0.5 RT, sec.	0.5 RT† /E.T.	% loss at peak	E.T., sec.	0.5 RT, sec.	0.5 RT /E.T.	Comment
27	72	68	60	78	1.3					
28	35	61	25	50	2.0					
30	79	70	155	1596	10.3					
30	27					87	24	30	1.25	
33	29	53	27	43	1.6	97	43	69	1.6	
43	125	49	215	151	.7	83	270	567	2.1	
31	51	56	21	103	4.9					
31	61	55	98	265	6.8					
28	63	80	145	1218	8.4					
27	38	44	24	38	1.6					
34	92	55	193	283	1.5	90	203	587	2.9	
30	56	49	39	113	2.9					Cephalic vein drainage
"	65	46	105	147	1.4					Median vein drainage
31	41	50	125	1375	11.0					Hand circulation only
40	67	40	160	224	1.4					*Idem*
36	35	56	33	92	2.8					Forearm only
45	114	67	220	418	1.9					*Idem*
33	102	61	160	224	1.4	87	185	518	2.8	"
32	32	56	36	209	5.8	91	85	196	2.3	"
38	70	48	146	‡						"
30	90	42	185	389	2.1					"
28	39	46	57	97	1.7	91	82	158	1.9	"
24	61	54	90	216	2.4	91	145	320	2.2	"
Mean	61	49	111	370	3.0	90	130	306	2 ± .6	
S.D.	±29	±19	±66	±199	±3.0	±4	±86	±256		

* E.T. = Equilibrium time. † 0.5 RT = Half-return time. ‡ Too long to calculate.
§ T_{T-1842} = Mean circulation time of T-1824.

the plasma (T-1824) minus 75 seconds. All of the values fell within the range of $3T_{T-1824}$-- (0 to 150 seconds).

Half-return time of thiocyanate. The half-return time of SCN was determined in each case according to the method outlined under "definition of terms," above. The mean half-return time for SCN in the 22 subjects was 370 ± 199 seconds (Table I). The period from appearance to half-return time averaged approximately 7 to 8 minutes. Since the equilibrium time represents the period during which net loss occurred it was of interest to compare it to the half-return time. In 20 cases the half-return time for thiocyanate ranged between 0.7 to 10.3 times the equilibrium or loss time with a mean of 3 ± 3.0 (Table I). Thus, even with a favorable concentration gradient the half return of this extracellular substance to the circulation averaged 3 times longer than the period of transcapillary loss.

Equilibrium and half-return times of heavy water. The great facility with which heavy water passes through semi-permeable membranes was shown not only by the early high percentage losses of this substance but also by the curves of expected and actual concentrations. In Fig. 2b and in most of the other cases as well the peak of the curve of actual concentrations occurred approximately 10 seconds later than the peak of the expected. This was interpreted as indicating a massive buildup of D_2O outside the capillaries at the time of peak passage of the bolus of injectate followed by rapid re-entry of a portion of this extravascular D_2O. This delayed peak of actual concentrations never was observed with SCN in the forearm circulation suggesting more hindrance to back diffusion of SCN than of D_2O. The larger space into which D_2O permeates was indicated by the some-

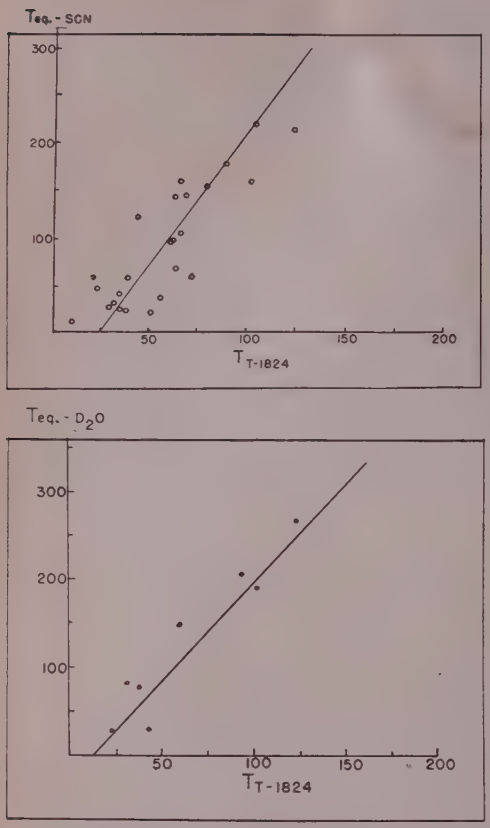

FIG. 3. (Upper graph.) The equilibrium times for thiocyanate are plotted against the respective mean circulation times of T-1824. The equation of the middle line is $T_{eq_{SCN}} = 3T_{T\text{-}1824}$ minus 75 seconds. (Lower graph.) Similar values for D_2O are plotted. The equation of this line is $T_{eq_{D_2O}} = 2.5$ $T_{T\text{-}1824}$ minus 31 seconds.

what longer equilibrium and half-return times for D_2O than for SCN. The equilibrium times of D_2O averaged 130 $\pm$ 86 seconds (Table I). These were related to the mean circulation times of the plasma as is indicated in Fig. 3b. Equilibrium time D_2O averaged 2.5 $T_{T\text{-}1824}$–31 seconds (range –7.5 to 70 seconds). In 7 cases SCN and D_2O were injected simultaneously. In these the equilibrium times D_2O averaged only 35 per cent more than the equilibrium times SCN. This again indicates the remarkable diffusion characteristics of heavy water through semi-permeable membranes since D_2O passes through cellular membranes and its extravascular space is approximately three times the SCN extravascular space.

In the 7 cases in which SCN and D_2O were injected simultaneously the half-return time of thiocyanate averaged 175 seconds and the half-return time of heavy water averaged 345 seconds. Thus, the mean half-return time D_2O was approximately twice as great as that of SCN.

The mean half-return time of D_2O in the 8 cases studied was 306 $\pm$ 256 seconds (Table I). This represented 2.1 $\pm$ 0.6 times the equilibrium time. Although these values are somewhat lower than the corresponding means for SCN, inspection of Table I will show that the latter were heavily weighted by a few cases with greatly prolonged half-return times. In all of the 8 cases the time from appearance to half-return of D_2O was less than 10 minutes.

Discussion. The validity of the method for determining percentage loss of the permeable tracers has been discussed previously (1). The method now has been extended to provide information on the rate of net exchange in both directions across the capillary membrane.

The deviations of the actual from the expected concentrations can only be explained by loss or gain to the circulating blood. Part of this loss could be into the walls of the blood vessels themselves which still would represent penetration into or through an endothelial membrane. It seems probable such losses are negligible compared to the transcapillary exchange.

The method does not provide information on the total exchanges in the forearm since the distribution of the labelled materials may not be evenly dispersed throughout all of the forearm vessels(5). However, since impermeable and permeable tracer substances are completely mixed prior to injection it is valid to assume that they will be delivered to the capillary beds of the area being sampled in the same relative concentrations as were present in the injectate.

The present state of knowledge regarding capillary permeability has been reviewed recently by Pappenheimer(6). Comparison of the present results with those reported previ-

ously in the literature is difficult because of differences in approach and technic. Much of the prior work has attempted to express the permeability characteristics of biological systems using the terminology employed in the physical sciences. The convention of using permeability constants representing the number of moles of a substance which cross unit cross-sectional area of the membrane in unit time under unit concentration difference may be useful in model systems where these variables are known. However, in biological systems they are not known and the attempt to estimate them introduces questionable assumptions and difficult complexities.

In regard to the organ systems of man we essentially are desirous of knowing (1) the magnitude of transcapillary loss of various substances and the factors which influence them, (2) the extravascular distribution of these substances, and (3) their "half life" in the tissues or their rate of return to the blood. Progress in this field especially in relation to the study of disease states depends upon the development of relatively direct and simple methods. Such methods need not mimic the approach used by the purely physical sciences where the experimental circumstances are completely different.

The net transcapillary loss of heavy water was approximately 90% and its return to the circulation was relatively rapid. The high rate of loss is consistent with previous observations from this laboratory and also with those of Chinard(7). Such high percentage losses and rapid returns are consistent with a process of diffusion rather than "filtration in bulk"(8). The half-return time of water was short when one considers that it also penetrates into cells. The present data suggest that more than half of the tissue water exchanges across the capillaries several times in an hour. It seems likely that this great movement which must include intracellular water serves as the medium through which much of the cellular metabolism is accomplished.

Thiocyanate met with more hindrance to free diffusion than did heavy water. This is consistent with the concept of restricted diffusion as advanced by Collander(9) and Weech and Michaelis(10) and further developed by Manegold(11) and Pappenheimer (12). The early percentage losses are less and its rate of return is no more rapid than D_2O despite the fact that it is limited to a space which is not only smaller but also is in more intimate contact with the capillaries. Nevertheless, turnover of SCN also is rapid since in most cases total net transcapillary loss was complete and half of this loss had returned to the circulation in less than 10 minutes from the time of injection.

The constant relationship between the mean circulation time of T-1824 and the equilibrium times of SCN and D_2O show that the net transcapillary exchanges of these permeable substances are blood flow dependent. Indeed, it is possible to predict the approximate equilibrium time of these substances when the rate of blood flow is known.

The present method offers several advantages over previously used technics for studying transcapillary exchange. The first is that the studies are conducted in normally functioning untraumatized tissue without use of artificial perfusion fluids. Only tracer doses of the injected materials are used which do not upset the osmotic equilibria of the blood in the capillaries under study. Another desirable feature is that sampling is carried out at intervals of seconds during the early phase. This is important since percentage losses must be determined during the brief period when the concentrations in the blood are higher than those in the tissues. The third advantage is the use of an impermeable tracer to cancel out the effects of dilution by the blood itself. The fourth is limitation of the study to a small vascular area in order to prevent significant recirculation of the tracer substances. As a result following completion of injection the blood entering the forearm will be practically free of labelled materials producing favorable concentration gradients for studying net rates of return of the substances under study. Thus, rate of net loss and rate of net return can be compared in a single experiment. Finally, the method is applicable to any organ or body area where an afferent

and efferent vessel are available for injection and sampling. A modification applicable to animals for preventing significant recirculation in organs with a large blood flow (such as the kidney) will be described in a subsequent communication.

Summary and conclusions. A method is presented for determining under physiological conditions the net bidirectional exchange of permeable tracer substances across the capillary walls of the human forearm. 1. In the early period, maximum net transcapillary loss for thiocyanate was 49 ± 19% and for deuterium oxide 90 ± 4%. 2. The equilibrium time or period of net loss averaged 111 ± 66 seconds for SCN and 130 ± 86 seconds for D_2O. Equilibrium times for both SCN and D_2O were related directly to blood flow. 3. The half-return time for SCN averaged 3 ± 3.0 times the equilibrium time. In the cases in which simultaneous measurements were made the half-return time of D_2O averaged twice that of SCN.

1. Freis, E. D., Higgins, T. F., and Morowitz, H. J., *J. Applied Physiol.*, 1953, v5, 526.

2. Lilienfield, L. S., Freis, E. D., Partenope, E. A., and Morowitz, H. J., *J. Clin. Invest.*, 1955, v34, 1.

3. Freis, E. D., Schnaper, H. W., Lilienfield, L. S., Wilson, I. M., and Kovach, R. D., *Circulation,* 1955, v12, 708.

4. Freis, E. D., Stanton, J. R., and Emerson, C. P., *Am. J. Physiol.*, 1949, v157, 153.

5. Andres, R., Zierler, K. L., Anderson, H. M., Stainsby, W. N., Cader, G., Chrayyib, A. S., and Lilienthal, J. L., Jr., *J. Clin. Invest.*, 1954, v33, 482.

6. Pappenheimer, J. R., *Physiol. Rev.*, 1953, v33, 387.

7. Chinard, F. P., Vosburgh, G. J., and Enns, T., *Am. J. Physiol.*, 1955, v183, 221.

8. Saperstein, L. A., Buckley, N. M., and Ogden, E., *ibid.*, 1955, v183, 178.

9. Collander, R., *Kolloid-Berhefte,* 1924, v19, 72.

10. Weech, A. A., and Michaelis, L., *J. Gen. Physiol.*, 1928, v12, 55.

11. Manegold, E., *Kolloid Z.*, 1937, v81, 164.

12. Pappenheimer, J. R., Renkin, E. M., and Borrero, L. M., *Am. J. Physiol.*, 1951, v167, 13.

Received April 9, 1956. P.S.E.B.M., 1956, v92.

Protective Effect of Pre-Irradiation on Lymphocytic Choriomeningitis Infection in Mice. (22425)

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X-irradiation has been found to affect the course of experimental viral infections in a variety of ways(1); in some instances the illness is aggravated(2-4), and in others it is ameliorated(5-8). It is the purpose of this communication to describe the striking beneficial effect of pre-irradiation on the course of lymphocytic choriomeningitis (LCM) infection of the adult mouse.

Materials and methods. Five-to 6-week-old white Swiss mice of the NMRI colony were used; the colony was carefully surveyed for the presence of latent LCM infection and no evidence of such was obtained. Guinea pigs were from the NMRI stock. Two strains of LCM were employed. The "Inst." strain is a mouse brain passaged strain which is uniformly lethal for mice after intracerebral inoculation. The "Arm." strain was recovered from house mice by Dr. C. Armstrong,‡ and maintained by 15 to 20 intraperitoneal passages prior to the experiments reported here; following inoculation by this route, it produces pleural and peritoneal effusion, pneu-

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† The opinions expressed are those of the author and do not necessarily represent those of the Bureau of Medicine and Surgery of the United States Navy.

‡ The "Inst." and "Arm." strains were received through the courtesy of Drs. E. Traub and C. Armstrong, respectively.

monitis, and some degree of hepatitis. The immunological identity of these strains was indicated by complete cross immunity in mice and guinea pigs, and by cross complement fixation reactions. Animals were subjected to single-exposure total body irradiation, using the technic of Ellinger, *et al.*(9), with 200 KVP and 25 ma., an HVL of 0.8 mm of copper, and an intensity of 43 r per minute. Mice received 300 r (LD_5), and guinea pigs 150 r. Infectivity titrations were carried out by intracerebral (i.c.) inoculation of 5 mice per 10-fold dilution, 0.04 ml per mouse; criteria of infection were typical cerebral symptoms or death 5 to 14 days after inoculation, or immunity to intracerebral challenge with approximately 10,000 LD_{50} of the Inst. strain, given 14-21 days after the initial inoculation. Fifty per cent end points were calculated by the Reed-Muench(10) method; titers are expressed in terms of 0.04 ml of a 10% organ suspension.

Results. Effect of pre-irradiation on meningitic form of the disease. Two chief experiments have been performed, using the same procedure. Half of a group of mice were irradiated, and 24 or 96 hours later equal numbers of irradiated and non-irradiated mice were injected i.c. with 0.04 ml of a dilution of a freshly prepared Inst. strain infected mouse brain suspension. Animals were examined daily or more often for the appearance of cerebral symptoms, by lifting and spinning by the tail, from the fifth through the twenty-first days after inoculation, and at less frequent intervals thereafter. Experimental details and the outcome of the infection in the irradiated and control groups are presented in Table I.

The infection was uniformly fatal for the non-irradiated mice; in the irradiated groups, however, the onset of the disease was delayed, symptoms were observed in less than half of the animals, and the course of the disease in symptomatic animals was not uniformly fatal. There was no apparent difference in the reaction of the groups inoculated one or four days after irradiation, or between the groups inoculated with greatly different doses of virus. While most of the irradiated mice did not develop cerebral symptoms of infection, they appeared ruffled and moderately emaciated from the second through the sixth week after the inoculation. In another experiment mice were challenged intracerebrally with approximately 10,000 LD_{50} of Inst. strain virus on the fifty-sixth day after irradiation; this group reacted identically with the non-irradiated controls. Thus, the beneficial effect of pre-irradiation had disappeared by the fifty-sixth day.

To compare the growth of the virus in the irradiated and non-irradiated mice, virus titers in the brain were determined at intervals in additional mice inoculated with the 10^{-4} dilution in Exp. 2, Table I. The titers

TABLE I. Effect of Total Body Irradiation (300 r) on Intracerebral Infection with LCM.

Exp. No.	Virus dilution	Irradiation	No. of mice inoculated	No. dying of cerebral symptoms	No. surviving cerebral symptoms	Late deaths*	Incubation period of cerebral symptoms Median	Range
1	10^{-4}	300 r‡	10	1	4	0	9	8-16
		0	11	11	0		6	6- 7
		300 r§	10	2	2	0	8	7-13
		0	10	10	0		6	5- 7
2	10^{0} †	300 r‡	10	2	0	2	9	9
		0	10	10	0		5	5- 7
	10^{-2}	300 r‡	10	2	2	1	10	8-14
		0	10	10	0		6	5- 7
	10^{-4}	300 r‡	14	4	2	2	12	9-15
		0	14	14	0		6	5- 7
Total		300 r	54	11	10	5	10	7-16
		0	55	55	0		6	5- 7

* Death between 12th and 75th days, not preceded by cerebral symptoms. † Titer—$10^{5.5}$ LD_{50} per 0.04 ml. ‡ Irradiated 24 hr before inoculation. § Irradiated 96 hr before inoculation.

TABLE II. Virus Content of Brains of Irradiated and Control Mice at Intervals after Intracerebral Inoculation.

Days after inoculation	Virus titer* in brains of individual mice	
	Irradiated	Non-irradiated
3	3.6, 3.5, 2.5	3.5, 3.6, 2.8
6	5.4, 5.5, 5.2	5.5, 5.5, 5.4
11	5.0, 5.5, 5.5	
16	4.5,† 4.8	
23	4.2, >5.3	
41	3.5, 4.5	
72	>3.5, 2.2	
157	>3.5, —‡	

* Titer expressed as negative log of ID_{50} dilution of .04 ml of 10% suspension.
† Convulsing at time of sacrifice.
‡ No virus detected.

obtained are recorded in Table II. The virus titers in the brains of the irradiated mice rose with the same rapidity and reached the same levels as in the non-irradiated group; in the former group the titers remained high for 2 to 5 months in some animals, and diminished or disappeared in others. No data are available for the persistence of virus following i.c. infection of non-irradiated animals with this strain of LCM since the disease is uniformly fatal in the doses employed in the present experiments.

Histological examination of the brains of asymptomatic irradiated mice sacrificed at intervals after virus inoculation showed minimal cellular infiltration of the basal meninges with no infiltration of the choroid plexus. Irradiated mice showing some degree of tremors or convulsions demonstrated a mild meningeal and choroid plexus infiltration which was much less severe than that of the non-irradiated controls.

Effect of irradiation on intraperitoneal form of the disease. Two experiments were carried out. Irradiated and non-irradiated mice were infected intraperitoneally with 0.1 ml of a dilution of a freshly prepared suspension of Arm. strain infected mouse spleen, 26 or 48 hours after irradiation. The mice were observed carefully for the appearance of the characteristic labored respiration which had been found to be an indication of pleural effusion(11), and mice dying were autopsied for confirmation of the presence of pleural fluid. On the seventh, ninth, or eleventh days after inoculation, surviving mice were sacrificed and the volume of fluid in the pleural and peritoneal cavities estimated with a one ml pipette equipped with a rubber bulb. One group of mice was set aside for observation without sacrifice. The findings are presented in Table III.

Production of serous exudation by the Arm. strain infection was completely prevented by the irradiation, regardless of the magnitude of the challenge inoculum. Although several of the irradiated mice died during the period of observation, none showed thoracic or peritoneal fluid, whereas all of the non-irradiated mice which died demonstrated large amounts of pleural exudate.

TABLE III. Effect of Pre-Irradiation on Intraperitoneal Infection with Arm. Strain LCM.

Exp.	Virus dilution	Irradiation	No. of mice	Signs of respiratory distress	Deaths	Day of sacrifice	Effusions in sacrificed mice*
3	10^{-3}	300 r†	14	1	0	11	0/14
		0	13	7	1		7/12
4	10^{0}	300 r‡	8	0	1	7	0/ 7
		0	15	13	6		8/ 9
	10^{-3}	300 r‡	12	0	0	9	0/12
		0	16	9	2		7/14
		300 r‡	13	1	3	Not	
		0	17	14	9	sacrificed	
Totals		300 r	47	2	4§		0/33
		0	61	43	18‖		22/35

* Numerator = No. of mice with more than 0.15 ml thoracic fluid. Denominator = No. of mice sacrificed. † Irradiated 26 hr before inoculation. ‡ Irradiated 48 hr before inoculation. § None had thoracic fluid at autopsy. ‖ All had abundant thoracic fluid at autopsy.

In the groups inoculated with the 10^{-3} virus dilution in experiment 4, three additional mice were sacrificed on the sixth day and the virus titer of the pooled liver, spleen, kidneys, and lungs of each mouse determined. The titers (ID_{50}) in the three irradiated mice were 4.7, 4.6, and 4.3, and in the non-irradiated mice, 5.3, 5.0, and 4.9. Thus, the virus titers in the irradiated group appeared to be slightly lower than in the controls.

In the groups of mice inoculated with the undiluted virus suspension in experiment 4, the livers and lungs of four of the sacrificed mice in each group were examined histologically. All 4 non-irradiated mice showed marked round-cell infiltration of the liver and moderate to severe interstitial pneumonia. On the other hand, the livers from the irradiated mice were normal, and the lungs showed no more than an occasional small area of pneumonic infiltration.

It should be noted that the survivors of the 10^{-3} infection in experiment 4 were challenged intracerebrally 55 days after infection, and both groups were found to be solidly immune. Thus, the modification of the infection by pre-irradiation did not interfere with the development of immunity.

Effect of irradiation on subcutaneous infection of the guinea pig. Five female guinea pigs weighing approximately 400 g were subjected to 150 r total body irradiation. Twenty-four hours later they and three normal guinea pigs of the same sex and weight were infected subcutaneously on the foot pad with 0.25 ml of a 10^{-2} dilution of a fresh 5% suspension of Arm. strain infected mouse spleen. In addition, two normal guinea pigs of approximately 800 g were inoculated with the same material.

All the animals in both the irradiated and non-irradiated groups died of the infection, and there was no significant difference in the times of death of the animals in the 2 groups (7 to 10 days in the irradiated guinea pigs, and 8 to 14 days in the controls).

Discussion. A number of factors are known to affect markedly the clinical reaction of mice to infection with LCM virus. Virus carriers, which may contain high titer virus throughout the body, show no outward response either to the carrier state or to massive virus challenge(12); normal suckling mice, while developing immunizing infection following virus inoculation, similarly demonstrate little or no clinical response to the infection(13). Immunized, non-carrier mice may support high titer proliferation of intracerebrally inoculated virus without showing any cerebral signs(11), and conversely, mice in the "accelerated stage" of immunity(14) may demonstrate cerebral signs at a time when the virus titer in the brain has risen but slightly(11).

The protective effect of irradiation on the meningitic form of LCM infection would appear to be entirely a suppression of reactivity to virus multiplication; despite growth of virus at the same rate as in non-irradiated animals, the irradiated animals generally showed neither clinical nor histological reaction to the virus. These findings are consistent with the hypothesis developed elsewhere (11) that the cerebral signs are a reaction to the presence of a meningeal infiltrate, which in turn is the result of host reactivity to the virus. Interpreted by this hypothesis, the protective effect of irradiation could be viewed as a result either of decreased tissue reactivity(15) or decreased ability of a leukopenic animal to form inflammatory exudates.

The effect of irradiation on the course of the intraperitoneally produced LCM infection was very marked, there being almost complete prevention of serous effusion and inflammatory reaction in the liver and lung. The relative importance of inhibition of viral multiplication, which was slight but probably significant, and of tissue reaction to infection will require further study.

Summary. 1. 300 r total body x-irradiation given to adult mice one to 4 days prior to intracerebral or intraperitoneal infection with LCM virus, produced a marked increase in survival and diminution in objective evidence of the infection. 2. The growth curve of the virus after intracerebral inoculation was identical in the irradiated and non-irradiated groups. After intraperitoneal infec-

tion with a viscerotropic strain there appeared to be a slightly lower virus titer in the pooled organs of the irradiated as compared with the non-irradiated mice.

1. Taliaferro, W. H., and Taliaferro, L. G., *J. Immunol.*, 1951, v66, 181.

2. Syverton, J. T., Werder, A. A., Friedman, J., Roth, F. J., Jr., Graham, A. B., and Mira, O. J., PROC. SOC. EXP. BIOL. AND MED., 1952, v80, 123.

3. Beutler, E., and Gezon, H. M., *J. Immunol.*, 1952, v68, 227.

4. Cheever, F. S., *ibid.*, 1953, v71, 431.

5. Le Fevre de Arric, M., *C. R. Soc. de Biol.*, 1927, v96, 208.

6. Goldberg, S. A., Brodie, M., and Stanley, P., PROC. SOC. EXP. BIOL. AND MED., 1935, v32, 587.

7. Baylin, G. J., Dubin, I. N., and Gobbel, W. G., Jr., *Am. J. Roentg. and Radium Ther.*, 1946, v55, 473.

8. Dubin, I. N., Baylin, G. J., and Gobbel, W. G., Jr., *ibid.*, 1946, v55, 478.

9. Ellinger, F., Morgan, J. E., Chambers, F. W., Biles, C. R., and Istock, J. T., *The Use of Small Laboratory Animals in Medical Radiation Biology.* Part I., Naval Med. Research Inst. Report NM 006 012.04.43, 30 April, 1952. Part II. NMRI Report NM 006 012.04.44, 15 May, 1952.

10. Reed, L. J., and Muench, H., *Am. J. Hyg.*, 1938, v27, 493.

11. Rowe, W. P., Studies on Pathogenesis and Immunity in Lymphocytic Choriomeningitis Infection of the Mouse, Naval Medical Research Institute Report NM 005 048.14.01, 27 May, 1954.

12. Traub, E., *J. Exp. Med.*, 1936, v63, 847.

13. Whitney, E., PROC. SOC. EXP. BIOL. AND MED., 1951, v78, 247.

14. Traub, E., *J. Exp. Med.*, 1938, v68, 229.

15. Becker, R. M., PROC. SOC. EXP. BIOL. AND MED., 1948, v69, 247.

Received April 12, 1956. P.S.E.B.M., 1956, v92.

Use of Anti-Rh Sera for Demonstrating Agglutination Activating Factor in Rheumatoid Arthritis.* (22426)

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There is a substance in the serum of most patients with rheumatoid arthritis that is capable of agglutinating previously unagglutinated, but sensitized red blood cells(1-4). This substance has been called(4) an agglutination activating factor (AAF). Not all red cells nor all anti-red cell antibodies are equally effective in demonstrating this factor. Ouchterlony and Jonsson(5) considered it necessary to use red cells from sheep or from animals closely related to sheep. However, Wager(4) was able to demonstrate AAF with human Group O cells or with the patient's own red cells sensitized with the corresponding rabbit antibody. The use of a human red cell sensitized with human antibody for the demonstration of this factor has not previously been reported.

Methods. 273 undiluted anti-Rh sera were used to sensitize equal volumes of a 2% suspension of washed Rh positive cells. After sensitization for one-half hour at 37° the cells were washed 3 times in saline and to the packed sensitized cells in duplicate tubes were added one volume of a 1:2 dilution of rheumatoid arthritis serum or of a normal control serum. After standing 10 minutes at room temperature, the cells were centrifuged at 1000 r.p.m. for one minute and the reactions read with a hand lens. Ninety-seven of the 273 anti-Rh sera proved to be unsuitable for these studies because of strong agglutination reactions developed by the anti-Rh antibody. Of the remaining 176, 30 provided sensitized cells which were strongly agglutinated by the rheumatoid serum, but not by the normal serum.

* This investigation was supported by research grant, PHS A-700, from Institute of Arthritis and Metabolic Diseases, N.I.H., Bethesda, and by Fellowship and Grant from the A. D. Williams Fund of the Medical College of Virginia.

TABLE I. Relation of Anti-Rh Titer to AAF Activity.

Anti-Rh titer (anti-globulin test)	No. of sera	No. positive
1: 4	33	0
1: 8	20	0
1: 16	30	3
1: 32	21	3
1: 64	31	8
1: 128	19	4
1: 256	9	5
1: 512	3	2
1:1024	5	2
1:2048	3	2
1:4096	2	1

Results. . As illustrated in Table I, there was no absolute relationship between the titer of anti-Rh antibody in the sensitizing anti-Rh serum, as measured by the indirect antiglobulin test, and agglutination of the sensitized cells by a rheumatoid arthritis serum. At titers greater than 1:128, however, over ½ the anti-Rh sera sensitized the cells to agglutination by the rheumatoid serum, while at 1:128 or below only ⅛ did so. The lack of absolute relation to antiglobulin titer is graphically illustrated in Table II, which shows in block titration the behavior of three high titer anti-Rh sera with a given rheumatoid arthritis serum.

For correlative studies with the sheep cell technic, sensitized Rh cells were prepared by adding an equal volume of washed 2% Rh positive cells to an anti Rh serum (Ri) diluted 1:10 with saline. The sensitized Rh cells were washed and added in 0.1 ml portions to equal volumes of rheumatoid arthritis sera titrated in saline. Controls included undiluted normal serum mixed with sensitized Rh cells and the rheumatoid serum mixed with unsensitized Rh cells. The sensitized sheep cell test was performed essentially as described by Heller(6), the sheep cells being sensitized with 1/20 the basic agglutinin titer of a rabbit anti-sheep cell serum and the titrations of inactivated rheumatoid arthritis sera being done in saline. The rheumatoid sera had been previously adsorbed with unsensitized sheep cells to remove heterophile antibody. No attempt was made to utilize sheep serum as an enhancing agent.

TABLE III. Titer of Rheumatoid Factor by Simultaneous Tests.

Patient	Sensitized sheep cells	Sensitized Rh cells
Br.	0	0
Ha.	0	0
Ca.	0	0
Har.	0	0
Tu.	8	0
Sp.	16	16
Je.	64	0
We.	16	128
Wo.	64	64
Pa.	64	512
Hu.	512	64
Zu.	256	256
Ed.	512	1024
No.	512	1024
Me.	1024	2048

In general, among 43 sera from various patients attending the arthritis clinic, titers with the Rh cell technic paralleled titers with the sheep cell technic. Fifteen illustrative examples are given in Table III. Certain outstanding discrepancies were noted, however, in which the titer was clearly greater by one method than by the other (Je, We, Pa). Seventy-six control sera taken at random from blood donors in the blood bank have been examined by the sensitized Rh cell technic and all but one found to give negative

TABLE II. Titer of Agglutination by a Rheumatoid Arthritis Serum or by a Coombs Serum for Cells Sensitized with Three Different Anti-Rh Sera.

Sensitizing serum (anti-Rh)	Agglutinating ("developing") serum	Anti-Rh serum, 1: 8	16	32	64	128	256	512	1024	2048	4096
Ba 16074	Rheumatoid	256	256	128	64	8	0	0	0	0	0
	Coombs	5120	2560	2560	1280	1280	1280	320	160	80	0
Mo 4-6	Rheumatoid	0	0	0	0	0	0	0	0	0	0
	Coombs	>5120	>5120	5120	5120	5120	2560	1280	640	160	80
Ri 3-53	Rheumatoid	256	256	256	256	128	64	8	0	0	0
	Coombs	>5120	>5120	>5120	>5120	5120	5120	5120	2560	2560	640

results.† The single exception was a healthy young mother, 2 weeks post partum from her third pregnancy.

Discussion. The finding that Rh positive cells sensitized with anti-Rh antibody may act as a suitable indicator system for the demonstration of AAF is in contrast to the experience reported by Wager(4). This worker, however, examined only a single anti-Rh serum. The fact that there exists a suitable immune system, involving only antibodies and antigens of human origin, for the demonstration of AAF is of interest in terms of the possible significance of this factor in the production of the disease process, rheumatoid arthritis.

Epstein, Johnson, and Ragan's recent finding(7) that AAF can be precipitated from rheumatoid arthritis sera by the addition of Fraction II in a typical precipitin reaction(8) has centered attention upon the probability that AAF is an antibody, not a complement-like material(2,9). The suggestion has been made(10) that AAF is an example of an auto-antibody. The present finding that certain anti-Rh sera are capable of sensitizing Rh positive cells so that they interact with AAF may be a manifestation of the Fraction II reaction described by Epstein, *et al.*(7). It is interesting that, just as Epstein, *et al.* (11), found a variation among various pools of Fraction II in their capacities to precipitate AAF, there is also a variation among anti-Rh sera in their capacities to sensitize an Rh (+) cell to agglutination by AAF.

The lack of strict correlation among various rheumatoid sera between the titers of activity against sensitized sheep cells and against sensitized Rh cells (Table III) suggests either that 2 distinct substances are involved, or that AAF is an antibody with various degrees of cross-reacting specificity for the sensitizing components in rabbit anti-sheep cell sera and in human anti-Rh sera.

It is not certain whether or not the Fraction II component which precipitates AAF is the same as the component in anti-Rh sera allowing suitable sensitization of Rh positive cells. More important, however, is whether or not any of the components studied, *i.e.* that in Fraction II, in anti-Rh sera, or in anti sheep cell sera, represents the antigen to which AAF is primarily directed. It is possible that all of these components are cross-reacting antigens, the primary antigen remaining as yet undetected. The finding (Je. Table III) that in some instances AAF activity against sensitized sheep cells exceeds that against sensitized Rh cells is consistent with the presumption that the anti-Rh serum component may not represent the primary antigen. Quantitative studies relating precipitable or adsorbable nitrogen(7,9) to agglutinating activity will be needed to clarify this subject.

Conclusions. It has been shown that certain anti-Rh sera can be utilized to sensitize Rh positive cells to the agglutination activating factor (AAF) in rheumatoid arthritis sera. The reaction depends upon some characteristic of anti-Rh sera other than titer alone. The possibility is supported that AAF may be a multicomponent system consisting either of multiple antibodies or of a single antibody with multiple cross specificities.

† In 3 instances, very weak reactions were encountered when non-inactivated sera were used. After 56° C for 30 minutes, these were entirely negative.

1. Cecil, R. L., Nicholls, E. E., and Stainsby, W. J., *Am. J. Med. Sci.*, 1931, v181, 12.

2. Dawson, M. H., Olmstead, M., and Boots, R. H., *J. Immunol.*, 1932, v23, 205.

3. Rose, H. M., Ragan, C., Pearce, E., and Lipman, M. O., PROC. SOC. EXP. BIOL. AND MED., 1948, v68, 1.

4. Wager, O., *Ann. Med. Exp. et Biol. Fenn.*, 1950, v28, suppl. 8.

5. Ouchterlony, O., and Jonsson, E., *Nord, Med.*, 1949, v42, 1449.

6. Heller, G., Jacobson, A. S., Kolodny, M. H., and Schuman, R. L., *J. Immunol.*, 1952, v69, 27.

7. Epstein, W., Johnson, A., and Ragan, C., PROC. SOC. EXP. BIOL. AND MED., 1956, v91, 235.

8. Kabat, E. A., and Mayer, M. M., *Experimental Immunochemistry*, Springfield, Ill., 1948, Charles C. Thomas.

9. Vaughan, J. H., *Ann. Rheum. Dis.*, 1955, v14, 431.

10. Steffen, C., and Schindler, H., *Schweis Z. f Bakt. u Path.*, 1955, v18, 287.

11. Epstein, W., Johnson, A., and Ragan, C., personal communication.

Received April 12, 1956. P.S.E.B.M., 1956, v92.

Inhibition of Brain Cytochrome Oxidase and ATP-ase by Chlorpromazine Analogues.* (22427)

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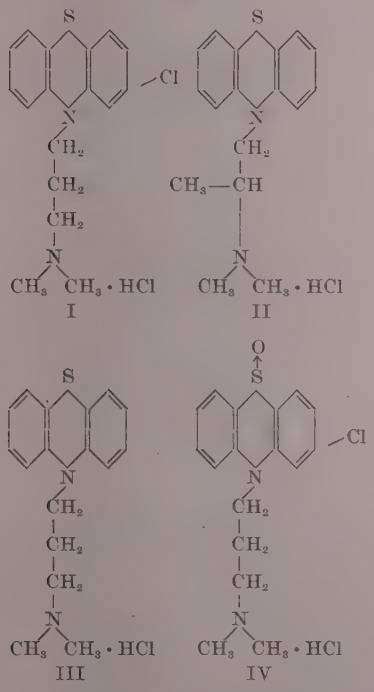
It has been previously reported that chlorpromazine inhibits brain cytochrome oxidase and ATP-ase systems *in vitro*(1,2). Apart from any mechanism of drug action which might be postulated by this inhibitory effect, it is of interest that 2 enzyme systems apparently unrelated, one involved in electron transport, the other a phosphatase, should both be similarly affected by the compound. Either the groupings involved on each of these enzymes bear a relationship to one another and allow them to react with the same unit on the chlorpromazine molecule, or 2 different groupings on the drug react with 2 differing centers on the enzymes.



To determine whether information on this problem could be elicited, 3 analogues of chlorpromazine (I), namely, promethazine (II), promazine (III), and chlorpromazine sulfoxide (IV) were studied in relation to their effect on the enzyme systems involved. Since the pharmacological activity of the drugs has been reported, some correlation between these activities and their inhibitory properties has also been explored.

Methods. Adult, albino, male rats, 200-300 g in weight, were exsanguinated and the brains removed and homogenized in 0.25 M sucrose without delay. A "mitochondrial" preparation was obtained by Procedure I of the method of Brody and Bain(3), and assayed for cytochrome oxidase and ATP-ase activity. The manometric assay of Schneider and Potter(4), was used for determining cytochrome oxidase using the standard Warburg procedure. ATP-ase levels were determined according to DuBois and Potter(5), using a Dubnoff metabolic incubator. ATP-ase activity was measured by analyzing for the phosphorus liberated during the experimental period by the method of Gomori(6). The Mg^{++}-activated enzyme was studied throughout. For cytochrome oxidase assays the inhibitor was placed in the main compartment and the enzyme tipped in from the side-arm, since previous studies had indicated that pre-incubation of enzyme and inhibitor did not alter the results. In the ATP-ase studies the enzyme was the final substance added to the reaction mixture. Cytochrome c was obtained from Sigma Chemical Company, and K_2ATP from the Pabst Laboratories. Chlorpromazine, promethazine and chlorpromazine sulfoxide were furnished by Dr. Leonard Cook of Smith, Kline and French Laboratories and promazine was donated by Dr. Daniel L. Shaw, Jr., of Wyeth Laboratories. The compounds were used as the hydrochlor-

* This investigation was supported in part by grant of Mental Health Fund of Illinois Department of Welfare.

TABLE I. Effect of Chlorpromazine Analogues on Cytochrome Oxidase Activity.

	Chlorpromazine		Promethazine		Promazine		Chlorpromazine sulfoxide	
Conc., M	μmoles/hr*	% inhibition	μmoles/hr	% inhibition	μmoles/hr	% inhibition	μmoles/hr	% inhibition
0	44.1		45.0		43.5		44.5	
1×10^{-4}	34.1	22.7	50.8	+12.9	48.8	+12.2		
5×10^{-4}	1.2	97.3	25.9	42.4	22.6	48.0	44.0	1.2
1×10^{-3}	0	100.0	7.8	82.7	4.1	90.5	41.8	7.2

* O_2 uptake/100 mg equivalent fresh tissue.

Reaction mixture: Cytochrome C, 8×10^{-5} M; Na ascorbate, 1.1×10^{-2} M; $AlCl_3$, 4×10^{-4} M; phosphate buffer, .04 M, pH 7.4; mitochondria equivalent to 20 mg fresh tissue. Inc. = 30 min.; F.V. = 3.0 ml.

ide. Experimental conditions for the assay are noted in the tables and the results for the analogues are averages of three experiments, while the chlorpromazine data are the averages of a number of determinations.

Results. The inhibitory properties of chlorpromazine toward cytochrome oxidase and ATP-ase are apparent from the data (Table I). In the cytochrome oxidase system, both promazine and promethazine evidence an inhibitory effect, though the two drugs are not as potent as chlorpromazine in this respect. The concentration of drug required to produce a 50% inhibition of this enzyme system is 2.5 x 10^{-4} M for chlorpromazine and 6.2 and 6.5 x 10^{-4} M for promazine and promethazine respectively. Chlorpromazine sulfoxide. on the other hand has no marked inhibitory effect on the system, the oxidation of the sulfur atom abolishing the cytochrome oxidase inhibition. The small activating effect of promazine and promethazine has also been observed for chlorpromazine at lower concentrations, but the significance of this finding is undetermined.

The inhibition of the ATP-ase system parallels the results obtained in the cytochrome oxidase assays (Table II). Chlorpromazine, promazine and promethazine all exert an effect and the concentration required to produce a 50% inhibition of this system is 4.1 x 10^{-4} M, 8.8 x 10^{-4} M and 10.7 x 10^{-4} M for the 3 drugs respectively. Again, as in the cytochrome oxidase system, the sulfoxide had no inhibitory effect. A comparison of the data shows that the cytochrome oxidase system is more sensitive to the action of the drugs studied, since the 50% inhibition values are lower in all cases for this enzyme system.

Discussion. Absence of any inhibitory effect of chlorpromazine sulfoxide toward ATP-ase and cytochrome oxidase systems, in contradistinction to other compounds studied, affords an opportunity to evaluate this mechanism as a factor in the pharmacological activity of the drugs. While some positive correlation is only suggestive, a negative relationship would eliminate this inhibitory mechanism as an explanation for the mode of action of the drugs. The use of chlorpromazine, promethazine and promazine as hypotensive agents(7), as potentiators of barbiturates(8), in sedation(9), and as ataractic drugs(10), has been well documented.

TABLE II. Effect of Chlorpromazine Analogues on ATP-ase Activity.

	Chlorpromazine		Promethazine		Promazine		Chlorpromazine sulfoxide	
Conc., M	μmoles/hr*	% inhibition	μmoles/hr	% inhibition	μmoles/hr	% inhibition	μmoles/hr	% inhibition
0	40.8		37.6		33.2		40.8	
1×10^{-4}	25.6	37.3	32.8	12.8	33.2	0		
5×10^{-4}	19.2	53.0	26.0	30.9	29.2	12.0	40.0	2.0
1×10^{-3}	10.8	73.5	16.4	56.4	17.2	48.2	39.6	2.9

* P liberated/100 mg equivalent fresh tissue.

Reaction mixture: K_2ATP, 3×10^{-3} M; $MgSO_4$, 3×10^{-3} M; veronal buffer, .023 M, pH 7.4; mitochondria equivalent to 40 mg fresh tissue. Inc. = 15 min.; F.V. = 2.6 ml.

Chlorpromazine sulfoxide is reported to have minimal sedation and potentiation effects in mice, rats and rabbits and only one-eighth the action of chlorpromazine in dogs. In addition its hypotensive properties in dogs are of a low order of magnitude(11). Effects of sulfoxide on mental state have not been reported. Thus it is evident that the sulfoxide derivative does not possess clinical properties of chlorpromazine, promazine and promethazine in areas which have been tested, and this parallels the findings on inhibition of cytochrome oxidase and ATP-ase *in vitro*. Since sulfoxide is a major metabolite of chlorpromazine(11), it seems that chlorpromazine is the active principle and does not act through its conversion to sulfoxide.

The prerequisite of a divalent sulfur in the ring might imply that other phenothiazine derivatives with an unsubstituted sulfur atom may also have the inhibitory properties reported. Collier and Allenby(12), have reported that neither phenothiazine, phenothiazine sulfoxide, phenothiazone nor thional were inhibitory to rat-liver cytochrome oxidase. Thus it would appear that substitution on the N atom of the ring might also be necessary to produce inhibition of this enzyme system.

Summary. 1. Inhibitory properties of 3 chlorpromazine analogues show that oxidation at the sulfur atom, abolishes inhibition of ATP-ase and cytochrome oxidase systems by chlorpromazine. 2. Removal of chlorine atom from the ring *viz*: promazine, and alteration of the N-substituted sidechain *viz*: promethazine, result only in a small diminution of the inhibitory properties. 3. Inhibitory findings are correlated with known pharmacological effects of the drugs.

1. Abood, L. G., PROC. SOC. EXP. BIOL. AND MED, 1955, v88, 688.

2. Bernsohn, J., Namajuska, I., and Cochrane, L. S. G., *Arch. Biochem. Biophys.*, in press.

3. Brody, T. M., and Bain, J. A., *J. Biol. Chem.*, 1952, v195, 685.

4. Schneider, W. C., and Potter, V. R., *ibid.*, 1945, v149, 217.

5. DuBois, K. P., and Potter, V. R., *ibid.*, 1943, v150, 185.

6. Gomori, C., *J. Lab. Clin. Med.*, 1942, v27, 955.

7. Rea, E. L., Shea, J., and Fazekas, J. F., *J.A.M.A.*, 1954, v156, 1249.

8. Kopera, J., and Armitage, A. K., *Brit. J. Pharm.*, 1954, v9, 392.

9. Baxter, R. W., Bolster, J. A., and McKechnie, S., *Anesthesia*, 1954, v9, 79.

10. Wyeth Laboratories, personal communication.

11. Salzman, N. P., Moran, N. C., and Brodie, B. B., *Nature*, 1955, v178, 1122.

12. Collier, H. B., and Allenby, G. M., *Can. J. Med. Sci.*, 1952, v30, 443.

Received April 12, 1956. P.S.E.B.M., 1956, v92.

Erythropoiesis. II. Assay of Erythropoietin in Hypophysectomized Rats.* (22428)

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Experimental evidence supporting the theory that a humoral factor in the blood plasma mediates red cell production has been reviewed by several investigators(1,2). This factor, which has been referred to as erythropoietin by Bonsdorff and Jalavisto(3) and others(4), has not been isolated nor is it known whether it is a single substance or several. The method utilized to determine the presence of erythropoietin in the plasma of anemic animals has been confined largely to evidence of increased erythrocyte production in normal animals that have received injections of plasma from animals made anemic by repeated phlebotomy or injections of phenylhydrazine. The hematologic technics for determining increased

* Number I in this series of papers appears in *J. Lab. Clin. Med.*, 1955, v46, 671.

red cell production consist largely of reticulocyte and red cell counts, hemoglobin or hematocrit determinations on the peripheral blood, and histologic study of erythroblastic activity of the blood-forming tissue. As previously reported(5,6), Fe^{59} uptake in newly-formed red cells is probably the most reliable single method for studying the effect of anemic plasma on erythropoiesis. Under ideal experimental conditions, the findings obtained by all these methods correlate well with one another, but an amount of plasma equivalent to one-half the blood volume is necessary to elicit a response. A sensitive method of assaying plasma from normal or anemic subjects is described in this communication.

Materials and methods. (a) Preparation of animals. Young adult male Sprague-Dawley rats, approximately 3 months of age and ranging in weight from 175 to 200 g, were used throughout these experiments. Rats were hypophysectomized by a standard technic† and delivered to us on the day following the operation or at later intervals thereafter. They were maintained on a diet consisting of milk, fresh vegetables, and Rockland mouse diet *ad libitum*. The control animals were of the same sex, age, and weight. *(b) Preparation of plasma.* Rats were bled daily for 3 days by cardiac puncture until their hematocrit was reduced to 25% or less. On the fourth day, the animals were anesthetized lightly with ether, and as much blood as possible was withdrawn by cardiac puncture. To this blood was added sufficient heparin to prevent clotting (circa 20 units per cc of blood). The blood was immediately centrifuged at 3000 rpm.‡ The plasma was separated, pooled with other plasma drawn at the same time, and stored at −10°C. This routine was followed for preparation of the plasma that we shall hereafter refer to as "anemic plasma." Plasma, which was prepared from the blood of normal, previously unbled rats, served as the control. *(c) Test of plasma for erythropoietin.* The hypophysectomized and normal rats were weighed just prior to and at the conclusion of each experiment. The plasma to be tested was administered intravenously to each animal in 2-cc doses on 3 consecutive days. Two hours following the last injection of plasma, 1 cc of Fe^{59} citrate solution, diluted with normal saline to contain 2 to 3 μc of radioactive Fe^{59}, was introduced into the tail vein. Sixteen hours following injection of Fe^{59}, a 1-cc sample of blood was withdrawn from each rat by cardiac puncture. The radioactivity in this sample was measured in a Nancy Wood well-type scintillation counter. The radioactivity in an aliquot of the original Fe^{59} solution given each animal was similarly measured. Using the blood volume of the rats (estimated to be 6% of body weight, verified by Cr^{51} blood volume determinations after the method of Gray and Sterling(7), the amount of radioactivity injected into the rats, and the radioactivity in the 16-hour sample, it is possible to calculate the percent of the injected dose of Fe^{59} appearing in the peripheral blood cells at this time interval. In each experiment, the percent of Fe^{59} taken up in a control group of rats (5 animals per group) was compared with the uptake in groups of rats receiving anemic plasma or normal plasma.

Results. (a) Effect of anemic plasma on uptake of Fe^{59} in hypophysectomized animals. Our first experiments were conducted on rats that were hypophysectomized 10 to 13 days prior to the injection of plasma. The uptake of Fe^{59} in hypophysectomized animals that were given daily injections of 2 cc each of normal plasma for 3 successive days was 5% or less; while in the hypophysectomized animals that were given anemic plasma, the uptake was 16% or more (Table I). The uptake

TABLE I. Effect of Anemic Plasma on Rate of Erythropoiesis in the Hypophysectomized Rat, 6 Experiments.*

No. rats per group	Avg % uptake of Fe^{59} into RBC — Anemic plasma	Normal plasma	Saline	No inj.
5	27.4	3.2	5.0	4.0
5	16.3	3.3		
4	17.1	5.0		
5	18	2.7	3.8	
3	19.3	5.0		
3	21.8	5.0		

* 10 to 15 days post-hypophysectomy.

† Obtained from Hormone Assay Laboratories, Chicago, Ill.

‡ International Centrifuge, size 1.

TABLE II. Effect of the Interval after Hypophysectomy on Apparent Sensitivity of Rat to Anemic Plasma.

Plasma inj.	Avg % Fe^{59} uptake into RBC of rats* Prior to hyp.†	4 days post-hyp.	8 days post-hyp.	13 days post-hyp.
Normal	37.5	9.1	4.0	5.0
Anemic	44.6	18.5	18.1	18.0

* 16-hr sample. † hyp. = hypophysectomy.

of Fe^{59} in normal rats following the injection of normal or anemic plasma according to the same regimen averaged respectively 37 ± 4% and 48 ± 4%. The data given below are from experiments in which hypophysectomized animals were used as recipients.

(b) Effect of interval post-hypophysectomy on senstivity to anemic plasma. A series of experiments was undertaken to determine at what time after hypophysectomy the animal is most sensitive to anemic plasma. Normal or anemic plasma was given to rats at various periods after hypophysectomy, and the rate of erythropoiesis was observed. The results are summarized in Table II. An appreciable reduction in Fe^{59} uptake occurred by 4 days after hypophysectomy and reached a minimum by 8 days. We found it advisable, therefore, to wait at least 8 days after surgery before using the animals for assay purposes.

(c) Effect of various amounts of anemic plasma on the uptake of Fe^{59}. Because of the greater sensitivity noted in the hypophysectomized rat, it was thought desirable to investigate the possibility of using a single injection of plasma, testing its efficacy when administered at various intervals prior to the injection of Fe^{59}. The data in Table III indicate that the erythropoietic effect induced by a single 2-ml injection of anemic plasma, given 4 hours prior to the Fe^{59}, is not manifest in the 16-hour sample, but is clearly seen when administered 24 hours prior to the administration of Fe^{59}. The stimulus is still detected 2 or 3 days after administration of plasma. These points were further substantiated by the observations that 2 doses of 2 cc each given 4 hours and 24 hours prior to Fe^{59} injection produced no greater effect on Fe^{59} uptake than did one injection given 24 hours previously. However, 2 injections, given 24 hours and 48 hours before Fe^{59}, gave a greater response. On the basis of these results, we elected to use 2 injections of 2 cc each in future assays unless otherwise noted.

TABLE III. Relationship of Interval between Single 2-ml Injection of Anemic Plasma and Administration of Fe^{59} to Elicitation of the Erythropoietic Stimulus; 5 Experiments.

Rats (No.)	Avg % uptake of Fe^{59} in RBC of hypophysectomized rats — Plasma given — 4 hr prior to Fe^{59}	24 hr prior to Fe^{59}	48 hr prior to Fe^{59}	Normal plasma
4		12.1		5.0
5		11.3	16.8	6.4
4		18.5	18.0	4.6
3		12.0	12.0	
10	4.5		15.5	4.0

(d) Production of anemic plasma factor by the hypophysectomized animal. Hypophysectomized animals were bled on 3 successive days until their hematocrit was 25% or less. Anemic plasma was obtained from these animals by the method that is described above. This anemic plasma, collected from hypophysectomized animals and injected into hypophysectomized animals, produced a significant increase in the uptake of Fe^{59} (Table IV). In other experiments it was found that plasma from normal animals subjected to splenectomy, adrenalectomy, thyroidectomy, gonadectomy, or combinations thereof and then bled increased the uptake of Fe^{59} in the hypophysectomized assay animal as well as did that from normal, bled animals.

Discussion. The data show that the anemic plasma factor(s) (erythropoietin) can be assayed in the hypophysectomized recipient using the technic of Fe^{59} incorporation into newly-formed red cells. The mechanism of the exaggerated response to anemic plasma observed in hypophysectomized animals as compared with the response of hypophysectomized animals given normal plasma is probably a relatively simple one. Our data indicate that by 4 days after hypophysectomy, erythropoiesis is already reduced but not to the maximum reduction, which occurs at 8 to 14 days. The reduction in erythropoiesis that we have shown to occur, using Fe^{59} uptake by the newly formed red cell as the criterion, has been corroborated by the finding of a reticulo-

TABLE IV. Effect of Hypophysectomy on Capacity of Animal to Elaborate the Anemic Plasma Factor.

Rats (No.)	Total vol of plasma, 2 cc/inj.	Avg % uptake of Fe^{59} in RBC of hypophysectomized rats after administration of various plasmas — Source of plasma: Unoperated, unbled animal	Hypophysectomized, unbled animal	Unoperated, anemic animal	Hypophysectomized, anemic animal
5	4	2.7	5.9	14.8	12.0
5	4	5.0	5.8	16.7	16.3
5	2			11.2	14.2
13	2			12.4	12.3

cyte reduction that coincides with the decreased uptake of Fe^{59}(8). It seems likely that an overall reduction in the metabolic requirements of the animal occurs very soon after hypophysectomy. Several workers(9, 10) have shown that a new equilibrium level of red cell mass is established within 2 or 3 months after hypophysectomy. Following the operation, for example, at day 10, the metabolic requirement of the animal is reduced but the new red cell mass equilibrium has not yet been achieved. The animal is therefore essentially comparable to an animal made polycythemic by transfusion. Consequently, red cell production falls to a minimum since a plethora of red cells already exists.

The production of erythropoietin probably also falls to a minimum under these circumstances, and, therefore, the administration of anemic plasma to the hypophysectomized animals produces an exaggerated response.

It is obvious from our data that the hypophysis is not directly concerned in the production of erythropoietin since plasma from an anemic hypophysectomized animal produces an excellent response in the hypophysectomized assay preparation. We need not enter into a discussion of whether a specific pituitary factor as suggested by Van Dyke *et al.*(10) and Contopoulos *et al.*(11) is involved directly in red cell production; but on the basis of the data reported in this paper and other data that have not yet been published, it seems likely that the effect of the pituitary on erythropoiesis is only indirect.

The great sensitivity of the hypophysectomized assay preparation to anemic plasma has made it possible for us to explore the presence of the factor(s) (erythropoietin) mediating red cell production in the plasma of normal rats and in other species with various types of anemia. These studies are being extended, and many other obvious facets of the problem, which are being investigated, will be reported at a later date.

Summary and conclusions. Using incorporation of Fe^{59} into newly-formed red cells as index of red cell production, it has been shown that erythropoiesis is gradually reduced, reaching a minimum in rats at 8 to 13 days after hypophysectomy. A factor of 10 exists between incorporation of Fe^{59} into red cells of normal control (37 ± 4) and the hypophysectomized rat (4 ± 2) at this interval. We have also found that the hypophysectomized rat is an extremely sensitive preparation for assay of factor(s) in anemic plasma that stimulates or mediates erythropoiesis. Our observations may be summarized as follows: 1. Administration of anemic plasma to hypophysectomized assay animal increases incorporation of Fe^{59} 3- to 7-fold. 2. Plasma from hypophysectomized animals and unoperated controls made anemic by repeated phlebotomy increases Fe^{59} red cell incorporation to the same extent when administered to the hypophysectomized assay animal. 3. A single injection of 2 ml of anemic plasma to the hypophysectomized assay animal elicits a 2- to 3-fold increase in Fe^{59} incorporation. The mechanism of rapid reduction of erythropoiesis that follows hypophysectomy in rats and its relationship to increased sensitivity of the hypophysectomized animal to anemic plasma are discussed briefly.

1. Grant, W. C., and Root, W. S., *Physiol. Rev.*, 1952, v32, 449.

2. Erslev, A. J., *Blood,* 1955, v10, 954.

3. Bonsdorff, E., and Jalavisto, E., *Acta Physiol. Scandinavica,* 1948, v16, 150.

4. Reisman, K. R., *Blood,* 1950, v5, 372.

5. Plzak, L. F., Fried, W., Jacobson, L. O., and Bethard, W. F., *J. Lab. Clin. Med.,* 1955, v46, 671.

6. Stohlman, F., Jr., and Brecher, G., PROC. SOC. EXP. BIOL. AND MED., 1956, v91, 1.

7. Gray, S., and Sterling, K., *J. Clin. Invest.,* 1950, v29, 1604.

8. Plzak, L., Fried, W., Jacobson, L. O., and Goldwasser, E., to be submitted for publication.

9. Meyer, O., Stewart, G. E., Thewlis, E. W., and Rusch, H. P., *Folia Haematol.,* 1937, v57, 99.

10. Van Dyke, D. C., Contopoulos, A. N., Williams, B. S., Simpson, M. E., Lawrence, J. H., and Evans, H. M., *Acta Haematologia,* 1954, v11, 203.

11. Contopoulos, A. N., Ellis, S., Simpson, M. E., Lawrence, J. H., and Evans, H. M., *Endocrinology,* 1954, v55, 808.

Received April 19, 1956. P.S.E.B.M., 1956, v92.

Adrenocortical Properties of $\Delta^{1,4}$-pregnadiene-17α,21-diol-3,11,20-trione (Meticorten) and $\Delta^{1,4}$-pregnadiene-11β,17α,21-triol-3,20-dione (Meticortelone).* (22429)

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The therapeutic usefulness of cortisone and hydrocortisone prompted a search for agents possessing anti-inflammatory actions similar to these steroids but producing a lower incidence of undesirable side effects. Efforts in these laboratories led to the development of two new steroids, Meticorten ($\Delta^{1,4}$-pregnadiene-17α, 21-diol-3,11,20-trione) and Meticortelone ($\Delta^{1,4}$-pregnadiene-11β,17α,21-triol-3,20-dione), which differ from cortisone and hydrocortisone in possessing an additional double bond between C_1 and C_2 in the A ring(1-3). Their effectiveness in the treatment of rheumatoid arthritis was first demonstrated by Bunim *et al.*(4).

The glucocorticoid potency of Meticorten and Meticortelone was found to be 3 to 4 times greater than that of cortisone and hydrocortisone by 3 different bioassay methods. Further comparison of the adrenocortical activities of the new steroids and their parent substances included 1) the life maintaining action in adrenalectomized rats and 2) the degree of hypercorticism induced in intact rats by prolonged steroid administration as well as the regression of these changes after cessation of treatment. The results of electrolyte excretion studies will be reported separately. Preliminary biological data have been reported previously(5,6). It is the purpose of this paper to present in detail the biological work carried out with Meticorten and Meticortelone.

Methods. 1. *Eosinophil response.* The procedure employed was based on the methods of Speirs and Meyer(7) and Rosemberg and co-workers(8). Steroids were screened in adrenalectomized C_{57} brown, male mice‡ at a dose of 24 μg without pretreatment with epinephrine. Preliminary potency estimates were performed with 3-fold dilutions of Meticorten and Meticortelone (6, 2, and 0.67 μg) and cortisone and hydrocortisone (12, 4, and 1.33 μg). The potency was calculated on

* Meticorten and Meticortelone, Schering Corp. brand of prednisone and prednisolone were used.

† The authors wish to thank Mrs. Shirley W. Bolles, Miss Janet Maley and Mrs. Alide Tali for valuable technical assistance. Mr. Gordon B. Thomas carried out statistical analysis of data, and members of the Chemical Research Division provided the steroids.

‡ Obtained from Roscoe B. Jackson Memorial Laboratory, Bar Harbor, Me. We wish to thank the members of our Pharmacology Department for performing the adrenalectomies.

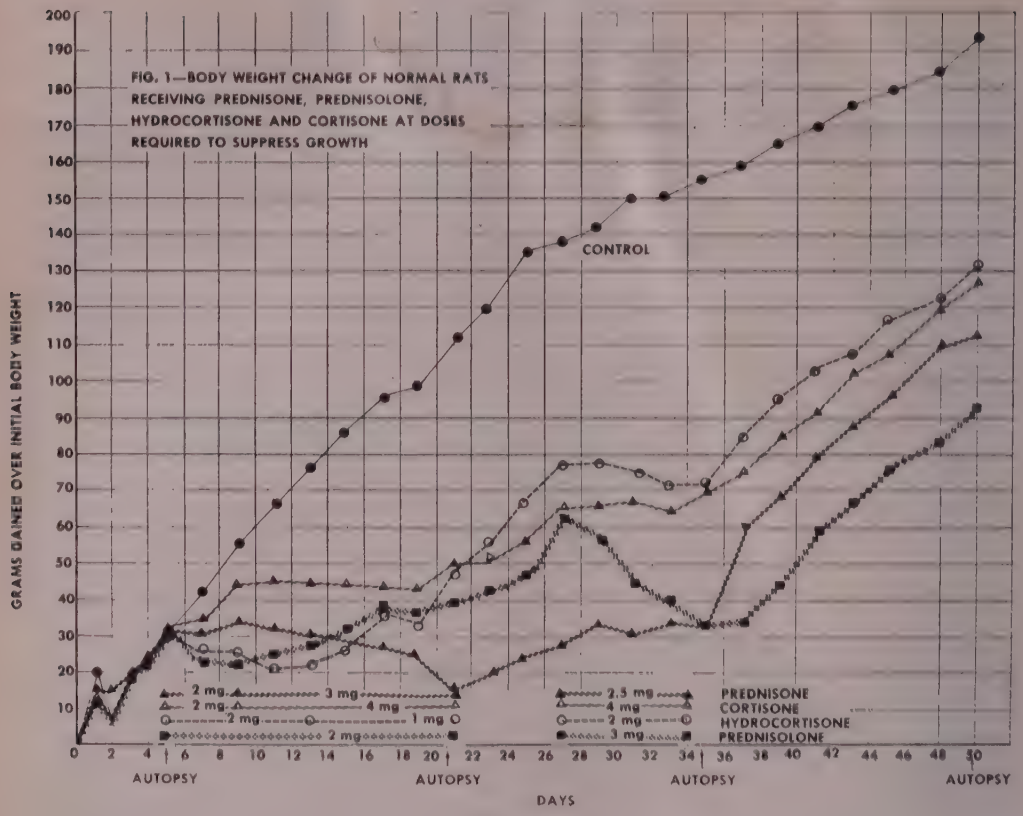

FIG. 1.

logarithmically transformed counts as described by Finney(9). 2. *Liver glycogen deposition.* Liver glycogen deposition was assayed in adrenalectomized male rats§ by the methods of Olson *et al.*(10) and Pabst *et al.*(11). Glycogen was estimated by the anthrone procedure of Kahan(12). 3. *Thymus involution.* The assay was patterned after that described by Stephenson(13) using intact, immature, female, litter-mate rats which had been rested for 1 week after shipment from the breeder. The steroids were dissolved in cottonseed oil and injected subcutaneously 3 times daily for 2 consecutive days.‖ On the morning of the third day, the animals were sacrificed and their thymi weighed to the nearest milligram. 4. *Life maintenance and growth.* Adrenalectomized, male rats weighing 50-70 g received 14 daily subcutaneous injections of Meticorten, Meticortelone, cortisone, hydrocortisone and desoxycorticosterone acetate (DCA) suspended in 1 part of ethanol and 9 parts of 50% aqueous propylene glycol. The animals were given tap water and Purina Lab Blox *ad libitum* and were weighed at 3-day intervals. The number of rats dying during each 24-hour period was tabulated until all completely operated animals had succumbed. Animals surviving for more than 3 weeks following the last injection and showing growth rates similar to those of the normal controls were eliminated from the experiment because of probable functional adrenal tissue. 5. *Effects of chronic administration of steroids on growth, organ weights and peripheral blood elements.* The degree of hypercorticism induced by pro-

§ All rats, both intact and adrenalectomized, were obtained from Charles River Breeding Laboratories, Brookline, Mass.

‖ Personal communication from Dr. Stephenson indicating this change in his published procedure.

longed steroid treatment and the reversibility of the resulting changes were studied in intact, growing rats. Suppression of somatic growth was selected as an easily defined physiological response and served as the basis for comparing the effects of the 4 steroids, Meticorten, Meticortelone, cortisone and hydrocortisone. Twenty-two groups of intact, male rats, weighing approximately 125 g, were housed 5 animals to a cage. They were maintained on stock Purina Chow diet and tap water and were weighed daily for the first 11 days and every other day thereafter. Each animal in a given cage received 1 of 5 treatments (Meticorten, Meticortelone, cortisone, hydrocortisone and diluent). After a 5-day observation period, the animals were injected subcutaneously daily for 19 days with saline suspensions of the appropriate steroid, rested for 3 days, and injected again daily for 8 days. The initial dose of 2 mg was adjusted periodically to insure the desired plateaued body weight. The injection schedule is indicated in Fig. 1. A 2-week recovery period was observed after the last injection. At various intervals, groups of animals were withdrawn from the experiment for autopsy. Tail blood was obtained before autopsy for the hematological examination, and an additional blood count was performed during the recovery period.

Results. Preliminary assays by the eosinophil method showed Meticorten to be 3.0 times as potent as cortisone (95% fiducial limits 1.6-5.4). Meticortelone exhibited 3.3 times the activity of hydrocortisone (95% fiducial limits 1.6-6.6). This potency ratio was confirmed by the liver glycogen deposition method (Table I) and by the thymus involution method (Table II). The results of the cross-potency assays were in satisfactory agreement with the values expected from the potency ratios of Meticorten and Meticortelone and their respective standards. In general, the results of the 3 bioassay methods were remarkedly concordant in spite of the relatively large inherent error of the individual assays.

In the life maintenance test (Table III), too, Meticorten and Meticortelone were approximately 4 times as potent as cortisone and hydrocortisone and seemed about as active as DCA. Meticorten and Meticortelone were, however, less effective than DCA in enhancing the body weights of immature, adrenalec-

TABLE I. Summary of Liver Glycogen Deposition Assays of Meticorten and Meticortelone in Adrenalectomized Male Rats.

Reference standard 100%	Substance assayed	Potency (%)	95% fiducial limits	Index of precision (λ)
Cortisone	Meticorten	326	190- 570	.44
		226	102- 457	.42
		260	125- 500	.48
		359	193- 665	.46
	W't'd mean*	286	214- 384	.45
	Meticortelone	1240	810-2120	.32
		829	461-1721	.46
	W't'd mean	1010	702-1456	.40
	Hydrocortisone	179	103- 310	.44
		248	164- 376	.31
	W't'd mean	220	158- 306	.38
Hydrocortisone	Meticortelone	422	225-1670	.30
		386	235- 658	.41
	W't'd mean	395	260- 599	.36
	Meticorten	220	144- 335	.30
		83	32- 165	.48
	W't'd mean	141	52- 380	.39
Meticorten	Meticortelone	231	128- 484	

* Weighted mean.

TABLE II. Summary of Thymus Involution Assays of Meticorten and Meticortelone in Immature Female Rats.

Reference standard 100%	Substance assayed	Potency (%)	95% fiducial limits	Index of precision (λ)
Cortisone	Meticorten	325	217- 577	.258
	Hydrocortisone	314	167- 629	.526
		255	179- 363	.298
	W't'd mean*	269	197- 367	
	Meticortelone	1410	940-2100	.319
		611	391- 956	.344
		900	600-1420	.376
	W't'd mean	923	570-1490	
Hydrocortisone	Meticortelone	164	46- 398	.750
		339	64-2330	1.012
		440	234- 859	.538
	W't'd mean	318	196- 515	
Meticorten	Meticortelone	174	110- 280	.288
		248	167- 359	.347
	W't'd mean	216	160- 292	

* Weighted mean.

TABLE III. Survival of Adrenalectomized, Immature Male Rats Treated for 14 Days with Meticorten, Meticortelone, Cortisone, Hydrocortisone and Desoxycorticosterone Acetate.

	Exp. I			Exp. II		
Daily dose, μg	Treatment*	No. of rats	Mean survival in days	Treatment*	No. of rats	Mean survival in days
180	DCA	13	21.5 ± 1.20†	DCA	14	21.4 ± .79†
90		18	13.7 ± 1.02		14	13.9 ± 1.19
45		17	9.4 ± .81		13	8.0 ± .76
480	F	11	19.5 ± .58	E	7	15.3 ± 1.26
240		11	14.9 ± .70		13	12.2 ± 1.09
120		10	13.0 ± 1.12		13	9.8 ± 1.09
120	Δ^1F	7	15.6 ± 1.29	Δ^1E	12	18.3 ± .68
60		9	14.1 ± 1.94		12	12.0 ± 1.14
30		10	10.3 ± 1.24		12	8.4 ± .57
15		9	8.0 ± .97			
—	C	5	8.2 ± 1.59	C	13	5.8 ± .44

* Treatment groups are indicated by the following letters: DCA = desoxycorticosterone acetate, F = hydrocortisone, Δ^1F = Meticortelone, E = cortisone, Δ^1E = Meticorten, C = controls.

† Stand. error of mean.

tomized rats. Twelve days after initiation of treatment, animals receiving 90 μg of DCA daily had gained 20 g whereas a daily dose of 120 μg of the 11-oxygenated steroids produced only the following weight increases: cortisone 7 g; hydrocortisone 12 g; Meticorten 17 g; and Meticortelone 12 g.

Changes in body weights observed during and after prolonged steroid treatment are plotted in Fig. 1. The daily dose required to suppress growth in young male rats may be estimated to be 4 mg for cortisone, 2.5 mg for Meticorten and 2 mg for hydrocortisone and Meticortelone. However, the effects of all 4 steroids on body weight were transient. This is clearly evident from the "escape" which occurred during the 3-day rest period and from the prompt resumption of growth at the termination of treatment.

The mean absolute and relative organ weights obtained at different intervals during the experiment are presented in Tables IV and V. All 4 steroids produced striking decreases in the absolute and relative weights of the thymus confirming the thymic involution observed in the short-term experiments described above. Meticorten and Meticortelone also suppressed the absolute and relative weights of the spleen, whereas in the cortisone and hydrocortisone groups, the spleen weights remained proportionate to the body weights. The absolute weights of the adrenals were more than 50% below the control values at the end of the injection period, in contrast to the slight reduction of the relative adrenal weights. All 3 organs resumed growth after cessation of treatment and showed signs of over-compensation 2 weeks after the last injection. The absolute weights of the liver, kidney, testes, seminal vesicles, thyroid and pituitary were only moderately decreased in the treated animals (Table IV), while the relative weights of these tissues actually exceeded those of the controls, with the exception of the seminal vesicles (Table V). In general, Meticorten and Meticortelone exerted a greater effect on organ weights than cortisone and hydrocortisone. Two weeks after cessation of treatment the organ weights had returned to essentially normal levels. Histological examination of the tissues revealed no pathological changes beyond the expected involution of lymphoid tissue and atrophy of the fascicular and reticular zones of the adrenal cortex.

The mean hematological data are presented in Table VI. The most striking change noted in the peripheral blood elements was the rapid and profound decrease in the percent of lymphocytes with the concomitant increase in polymorphonuclear leucocytes in the animals treated with Meticorten and Meticortelone, and to a lesser degree, in the cortisone and hydrocortisone groups. The total number of

TABLE IV. Absolute Organ Weights for Groups of Male Rats Treated with Meticorten, Meticortelone, Cortisone and Hydrocortisone.

Treatment*	Body wt (g)	Spleen (g)	Liver (g)	Kidney (g)	Adrenals (mg)	Testes (g)	Thymus (g	Seminal vesicles (g)	Pituitary (mg)	Thyroids (mg
C‡	163 ± 4.37†	.702 ± .139	7.27 ± .221	1.50 ± .005	27.7 ± 1.17	1.99 ± .073	.50 ± .25	.194 ± .018		
△¹E§	149 ±13.7	.482 ± .087	7.95 ± .468	1.71 ± .079	14.4 ± .758	2.43 ± .073	.043 ± .0053	.464 ± .100	9.50 ± .634	
E	193 ± 6.41	.878 ± .064	8.52 ± .461	1.82 ± .040	25.8 ± 4.86	2.44 ± .157	.134 ± .014	.730 ± .056	10.60 ± .52	
F	180 ± 7.08	.716 ± .132	7.66 ± .374	1.80 ± .066	22.6 ± 1.99	2.61 ± .096	.174 ± .014	.436 ± .065	9.62 ± .63	
△¹F	161 ± 7.92	.520 ± .118	7.94 ± .564	1.68 ± .081	20.6 ± 3.29	2.43 ± .114	.114 ± .012	.424 ± .060	8.08 ± .77	
C	237 ± 6.58	1.09 ± .233	9.26 ± .287	1.96 ± .088	33.9 ± 1.94	2.74 ± .104	.520 ± .028	.670 ± .064	11.54 ± 1.68	
△¹E¶	152 ± 8.76	.56 ± .101	8.19 ± .387	1.78 ± .071	14.4 ± 2.49	2.50 ± .039	.078 ± .022	.516 ± .093	9.7 ± 1.80	15.5 ± 2.08
E	210 ±21.29	.80 ± .110	10.10 ± .911	2.14 ± .143	16.9 ± 1.63	2.47 ± .139	.186 ± .001	.658 ± .129	8.9 ± 1.68	15.7 ± 1.76
F	199 ± 3.45	.78 ± .144	9.49 ± .445	1.88 ± .062	15.6 ± 1.50	2.74 ± .092	.117 ± .018	.520 ± .034	6.7 ± .75	16.1 ± 2.67
△¹F	159 ±11.19	.35 ± .050	8.79 ± .797	1.92 ± .146	17.5 ± 1.91	2.60 ± .071	.036 ± .006	.348 ± .034	7.1 ± .45	12.1 ± 1.29
C	287 ± 6.69	1.22 ± .339	11.28 ± .438	2.71 ± .767	33.3 ± 2.71	3.00 ± .047	.286 ± .068	.822 ± .130	9.9 ± .22	19.9 ± 1.96
△¹E¶	240 ± 9.48	1.36 ± .138	10.59 ± .792	2.46 ± .115	30.9 ± 2.19	2.77 ± .060	.504 ± .022	.844 ± .051	10.7 ± .89	19.0 ± 2.70
E	247 ±15.76	1.48 ± .104	10.78 ± .865	2.29 ± .283	39.9 ± 4.44	2.86 ± .233	.542 ± .051	.934 ± .089	10.4 ± .90	17.46 ± 5.57
F	238 ±16.35	1.56 ± .157	9.81 ± .610	2.36 ± .054	30.8 ± 2.12	2.89 ± .177	.514 ± .060	.726 ± .102	9.7 ± .99	20.66 ± 1.55
△¹F	236 ± 8.63	1.82 ± .204	12.90 ± .656	2.42 ± .137	32.3 ± 3.76	2.92 ± .137	.434 ± .048	1.00 ± .066	9.24 ± .54	16.8 ± 1.80
C	312 ±12.14	1.52 ± .158	12.41 ± .557	2.80 ± .143	43.6 ± 3.01	3.13 ± .169	.586 ± .062	1.02 ± .054	12.24 ± .63	21.44 ± 1.19

* Treatment groups are indicated by following letters: C = controls, △¹E = Meticorten, E = cortisone, F = hydrocortisone, △¹F = Meticortelone.
† Means and stand. dev. of mean for 10 rats/group on day 5 and for 5 rats/group for subsequent autopsies. ‡ Autopsied at start of inj. § Autopsied on day 21 at end of inj. ¶ Autopsied on day 35 at end of inj. ‖ Autopsied on day 50 at end of 2 wk recovery period.

TABLE V. Organ Weights per 100 g Body Weight for Groups of Male Rats Treated with Meticorten, Meticortelone, Cortisone and Hydrocortisone.

Treatment*	Spleen (g	Liver (g	Kidney (g	Adrenals mg	Testes g	Thymus g	Seminal vesicles g	Pituitary mg	Thyroids mg
C‡	.433 ± .064†	4.47 ± .128	.918± .018	16.9 ± .432	1.26 ± .053	.306 ± .015	.119 ± .008		
△¹E§	.324 ± .047	5.49 ± .471	1.17 ± .073	10.1 ± 1.22	1.72 ± .210	.029 ± .007	.308 ± .063	6.0 ± .61	
E	.523 ± .046	4.40 ± .127	.94 ± .027	10.6 ± 2.85	1.26 ± .068	.069 ± .008	.312 ± .039	5.0 ± .37	
F	.409 ± .103	4.26 ± .113	1.00 ± .040	10.5 ± 1.34	1.47 ± .083	.098 ± .010	.245 ± .039	5.0 ± .31	
△¹F	.326 ± .074	4.99 ± .412	1.06 ± .066	11.5 ± 1.56	1.53 ± .118	.068 ± .007	.258 ± .028	5.0 ± .44	
C	.452 ± .091	3.93 ± .094	.83 ± .027	14.3 ± .735	1.17 ± .056	.222 ± .013	.238 ± .018	5.0 ± .61	
△¹E¶	.39 ± .051	5.44 ± .210	1.18 ± .025	9.6 ± 1.61	1.67 ± .079	.053 ± .014	.35 ± .075	6.2 ± .85	10.4 ± 1.01
E	.38 ± .063	4.80 ± .099	1.03 ± .030	8.2 ± .78	1.20 ± .059	.083 ± .022	.29 ± .046	3.7 ± .62	7.5 ± .66
F	.39 ± .078	4.76 ± .262	.94 ± .039	7.8 ± .75	1.37 ± .042	.057 ± .009	.26 ± .019	3.3 ± .35	8.0 ± 1.19
△¹F	.22 ± .036	5.50 ± .138	1.25 ± .181	11.1 ± 1.02	1.67 ± .079	.022 ± .0027	.22 ± .041	4.6 ± .041	7.9 ± 1.13
C	.42 ± .116	3.95 ± .221	.95 ± .092	11.7 ± 1.04	1.05 ± .031	.118 ± .015	.28 ± .042	3.5 ± .026	6.9 ± .34
△¹E¶	.57 ± .063	4.44 ± .356	1.03 ± .057	12.6 ± .519	1.16 ± .046	.21 ± .014	.35 ± .022	4.5 ± .299	7.9 ± .87
E	.62 ± .086	4.08 ± .190	.92 ± .095	16.2 ± 1.49	1.16 ± .062	.22 ± .019	.37 ± .021	4.2 ± .255	6.6 ± 1.84
F	.67 ± .084	4.10 ± .134	1.01 ± .052	13.0 ± .658	1.21 ± .009	.22 ± .033	.31 ± .050	4.0 ± .340	8.7 ± .50
△¹F	.77 ± .076	4.62 ± .178	1.02 ± .036	13.7 ± 1.34	1.25 ± .081	.18 ± .021	.43 ± .025	3.8 ± .177	7.2 ± .65
C	.49 ± .057	3.97 ± .077	.90 ± .042	14.7 ± .754	1.04 ± .056	.19 ± .019	.34 ± .017	4.1 ± .404	7.1 ± .38

* Treatment groups are indicated by following letters: C = controls, △¹E = Meticorten, E = cortisone, F = hydrocortisone, △¹F = Meticortelone.
† Means and stand. dev. of mean for 10 rats/group on day 5 and for 5 rats/group for subsequent autopsies. ‡ Autopsied at start of inj. § Autopsied on day 21 at end of inj. ¶ Autopsied on day 35 at end of inj. ‖ Autopsied on day 50 at end of 2 wk recovery period.

TABLE VI. Changes in Peripheral Blood Elements of Male Rats Treated with Meticorten, Meticortelone, Cortisone and Hydrocortisone.

Treatment*	Hematocrit, %	Total WBC/mm³	Lymphocytes, %	Polymorphonuclear leukocytes
C‡	47.5 ± .864†	20,850 ± 2,083	78.9 ± 1.63	18.6 ± 1.576
Δ^1E§	54.4 ± 1.641	16,520 ± 2,478	46.8 ± 5.19	52.5 ± 4.74
E	59 ± 3.74	15,975 ± 1,669	73 ± 1.64	25 ± 1.80
F	60 ± 4.93	16,750 ± 1,286	66 ± 8.26	32.4 ± 7.94
Δ^1F	51.2 ± 1.11	23,140 ± 6,358	40.4 ± 7.7	58.4 ± 8.14
C	61.5 ± 4.53	19,620 ± 493	79.6 ± 2.34	14.6 ± 1.51
Δ^1E‖	49.6 ± 1.00	21,250 ± 1,448	41 ± 11.94	58.8 ± 10.77
E	50 ††	14,484 ± 1,178	65 ± 7.00	34 ± 7.00
F	50 ††	19,100 ± 4,843	58.4 ± 8.22	50.8 ± 6.98
Δ^1F	51 ††	16,290 ± 2,803	10 ± 1.60	90 ± 1.60
C	49 ††	18,400 ± 1,385	81.2 ± 2.32	16.4 ± 2.35
Δ^1E¶	45.4 ± 1.08	20,870 ± 4,445	73.2 ± 4.77	25.8 ± 4.86
E	47.6 ± 1.46	20,110 ± 2,669	80.4 ± 2.81	17.6 ± 1.95
F	49.6 ± .61	14,190 ± 3,390	58.4 ± 10.25	39.6 ± 10.36
Δ^1F	45.4 ± .73	13,420 ± 1,336	60.6 ± 6.60	38.4 ± 7.11
C	49.6 ± .54	20,370 ± 1,949	78.6 ± 4.72	20.4 ± 4.53
Δ^1E**	46.6 ± 1.91	15,340 ± 2,636	79.4 ± 4.12	19.2 ± 3.99
E	53.6 ± 1.89	17,390 ± 1,765	80.4 ± 5.35	17.6 ± 4.93
F	52.4 ± 1.25	15,980 ± 1,502	79.6 ± 4.28	20.4 ± 4.41
Δ^1F	53.0 ± 1.72	16,500 ± 1,909	63.8 ± 4.54	33.8 ± 4.22
C	49.4 ± .36	20,080 ± 6,444	79.2 ± 3.90	18.6 ± 3.39

* Treatment groups are indicated by following letters: C = controls, Δ^1E = Meticorten, E = cortisone, F = hydrocortisone, Δ^1F = Meticortelone.

† Means and stand. dev. of mean for 10 rats/group on day 5 and for 5 rats/group for subsequent bleedings.

‡ Bled at start of injections. § Bled on day 21. ‖ Bled on day 35 at end of inj. ¶ Bled on day 42 one wk after last inj. ** Bled on day 50 2 wk after last inj. †† Means for less than 5 rats.

leukocytes was variable, and the hematocrit did not show any outstanding or consistent changes. It is of interest that the inverted lymphocyte/polymorphonuclear leukocyte ratio began to return towards normal values 1 week after cessation of treatment. Two weeks after the last injection, all but the Meticorten-treated animals had normal blood counts.

Discussion. The introduction of a second double bond in the A ring of cortisone and hydrocortisone produced a 3- to 4-fold increase in the glucocorticoid activity of Meticorten and Meticortelone. Since completion of our experiments, similar findings have been reported for Meticortelone by Stafford *et al.* (14) and for Meticorten by Ferrari *et al.*(15). Increases in glucocorticoid activity also were observed in this laboratory for Δ^1-corticosterone and Δ^1-desoxycorticosterone. Compared to cortisone (100%) by the liver glycogen assay method, the following activity ratios were found: Δ^1-corticosterone, 294% (95% fiducial limits 197-440); corticosterone, 119% (80-177); Δ^1-desoxycorticosterone, 7.8% (4.9-12.4); desoxycorticosterone, 1.8% (1.1-2.7). Potentiation of the biological activity of compounds possessing the $\Delta^{1,4}$-configuration had been noted previously by Huggins *et al.*(16) in the androstadiene series. They found $\Delta^{1,4}$-androstadiene-3, 17-dione more effective in promoting uterine growth in hypophysectomized rats than either the Δ^1 or Δ^4 analogues.

Unsaturation in both the A and B rings of cortisone apparently does not enhance glucocorticoid activity. Thus the Δ^6 analogue of cortisone acetate was found in this laboratory to possess only 53% (fiducial limits 39-78) of the activity of cortisone acetate in the liver glycogen assay. This observation concurs with the studies of this compound by Higgins *et al.*(17).

Glucocorticoid activity greatly exceeding that of the natural hormones and of Meticorten and Meticortelone has been demonstrated for 9α-halogenated steroids(18), and for steroids methylated at C_2 or possessing both

modifications(19). However, both types of chemical alterations, alone or in combination, resulted in greatly enhanced mineralocorticoid potency(19,20) whereas Δ^1-dehydrogenation did not affect electrolyte activity. In unpublished experiments in this laboratory, it was found that Meticorten and Meticortelone failed to produce sodium retention in adrenalectomized rats. Similar findings have been reported recently for both steroids(14,21) as well as for Δ^1-desoxycorticosterone(22). Likewise, the introduction of an additional double bond between C_1 and C_2 in 9α-fluorohydrocortisone greatly enhanced glucocorticoid but not mineralocorticoid activity(14, 23).

Meticorten and Meticortelone were more active than their parent substances in maintaining the life of young, adrenalectomized rats (Table III). This observation is at variance with that of Swingle¶ who found that Meticorten and Meticortelone had only one-fourth the activity of cortisone and hydrocortisone in maintaining adrenalectomized dogs. Similar species differences between rats and dogs have been reported in life maintenance studies with 9α-halogenated corticoids(20, 24). It will be noted that the daily amounts of steroids required to maintain our rats were considerably higher than the doses of cortisone used by Leatham and Wolf(25). This difference may be attributed to the high protein diet employed by these investigators which is known to have a beneficial effect on the survival of adrenalectomized rats.

The manifestations of hypercorticism induced by prolonged steroid administrations to intact rats were more marked in the Meticorten and Meticortelone groups than in the animals treated with cortisone and hydrocortisone. The suppression of growth and the changes in organ weight during treatment and their subsequent return to normal values were in general agreement with the findings obtained with cortisone acetate(26-28) and with Meticorten(15). The fact that in the present study the relative adrenal weights were not suppressed to the same extent as reported in the literature may be attributed to differences in dose, length of treatment, initial body weight of the animals and to the pair-feeding of the controls carried out by most investigators.

Higgins and co-workers(17) noted a fall in the total leukocyte count of 10,300 cells per cu mm during cortisone treatment, the control count being in the neighborhood of 16,000. As shown in Table VI, the total white cell count of our controls ranged from 18,400 to 20,840; and the lowest count observed in the treated rats was 13,420 in the Meticortelone group 1 week after the last injection. No explanation for this discrepancy can be offered.

Summary. (1) Meticorten and Meticortelone, 2 new steroids, were studied in animals to assess their adrenocortical activities. (2) Glucocorticoid properties were assayed by the eosinophil response in adrenalectomized mice, by the liver glycogen deposition in adrenalectomized rats, and by the involution of the thymus in immature rats. (3) By all 3 methods, the glucocorticoid activity of Meticorten and Meticortelone was found to be 3 to 4 times greater than that of cortisone and hydrocortisone. (4) Meticorten and Meticortelone were more active than cortisone and hydrocortisone in maintaining the life of immature, male, adrenalectomized rats. (5) Prolonged administration of Meticorten, Meticortelone, cortisone and hydrocortisone to young, male rats produced the following biological changes: a) suppression of somatic growth, b) decrease in thymus, spleen and adrenal weights on an absolute basis as well as per 100 g body weight, c) increase in the relative weights of liver, kidney, testes, thyroid and pituitary and inconsistent changes in the weight of the seminal vesicles, d) inversion of the lymphocyte/polymorphonuclear leukocyte ratio in the circulating blood without consistent changes in total white cell count or hematocrit. (6) Meticorten and Meticortelone were more potent than cortisone and hydrocortisone in affecting organ weights and peripheral blood elements. (7) The changes induced by prolonged administration of the 4 steroids were readily reversible after cessa-

¶ We wish to thank Dr. Swingle for making the results of his study available.

tion of treatment.

1. Herzog, H. L., Nobile, A., Tolksdorf, S., Charney, W., Hershberg, E. B., Perlman, P. L., and Pechet, M. M., *Science,* 1955, v121, 176.

2. Nobile, A., Charney, W., Perlman, P. L., Herzog, H. L., Payne, C. C., Tully, M. E., Jevnik, M. A., and Hershberg, E. B., *J. Am. Chem. Soc.,* 1955, v77, 4184.

3. Herzog, H. L., Payne, C. C., Jevnik, M. A., Gould, D., Shapiro, E. L., Oliveto, E. P., and Hershberg, E. B., *ibid.,* 1955, v77, 4781.

4. Bunim, J. J., and Pechet, M. M., *J. Am. Med. Assn.,* 1955, v157, 311.

5. Perlman, P. L., and Tolksdorf, S., *Fed. Proc.,* 1955, v14, 377.

6. Tolksdorf, S., and Perlman, P. L., *Endocrine Society Meeting,* June 2-4, 1955, 37th Meeting.

7. Speirs, R. S., and Meyer, R. K., *Endocrinology,* 1951, v48, 316.

8. Rosemberg, E., Cornfeld, J., Bates, R. W., and Anderson, E., *ibid.,* 1954, v54, 363.

9. Finney, D. J., *Statistical Method in Biological Assay,* 1952, Charles Griffin and Co., London.

10. Olson, R. E., Jacobs, F. A., Richert, D., Thayer, S. A., Kopp, L. J., and Wade, N. J., *Endocrinology,* 1944, v35, 430.

11. Pabst, M. L., Sheppard, R. S., and Kuizenga, M. H., *ibid.,* 1947, v41, 55.

12. Kahan, J., *Arch. Biochem. and Biophys.,* 1953, v47, 408.

13. Stephenson, N. R., *Canad. J. Biochem. and Physiol.,* 1954, v32, 689.

14. Stafford, R. O., Barnes, L. E., Bowman, B. J., and Meinzinger, M. M., PROC. SOC. EXP. BIOL. AND MED., 1955, v89, 371.

15. Ferrari, V., Ginoulhiac, E., Mosca, M. C., and Tenconi, L. T., *Minerva Medica,* 1955, v46, 51.

16. Huggins, C., Jensen, E. V., and Cleveland, A. S., *J. Exp. Med.,* 1954, v100, 225.

17. Higgins, G. M., Woods, K. A., and Kendall, E. C., *Endocrinology,* 1951, v48, 175.

18. Fried, J., and Sabo, E. F., *J. Am. Chem. Soc.,* 1953, v75, 2273.

19. Byrnes, W. W., Barnes, L. E., Bowman, B. J., Dulin, W. E., Morley, E. H., and Stafford, R. O., PROC. SOC. EXP. BIOL. AND MED., 1956, v91, 67.

20. Borman, A., Singer, F. M., and Numerof, P., *ibid.,* 1954, v86, 570.

21. Chart, J. J., Hetzel, N., and Gaunt, R., *ibid.,* 1956, v91, 73.

22. Nairn, R. C., Masson, G. M. C., and Corcoran, A. C., *Endocrinology,* 1955, v57, 700.

23. Hirschman, R., Miller, R., Beyler, R., Sarett, L., and Tishler, M. T., *J. Am. Chem. Soc.,* 1955, v77, 3166.

24. Swingle, W. W., Baker, C., Eisler, M., Le Brie, S. J., and Brannick, L. J., PROC. SOC. EXP. BIOL. AND MED., 1955, v88, 193.

25. Leatham, J. H., and Wolf, R. C., *ibid.,* 1954, v86, 724.

26. Migeon, C. J., Gardner, L. T., and Crigler, J. F., *Endocrinology,* 1952, v51, 117.

27. Halmi, N. S., and Barker, S. B., *ibid.,* 1952, v51, 127.

28. Bodansky, O., and Money, W. L., *ibid.,* 1954, v55, 173.

Received April 24, 1956. P.S.E.B.M., 1956, v92.

Distribution of Fetal Hemoglobin in Layers by Centrifugation. (22430)

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Hemoglobin of the fetal blood is of 2 varieties, that can be differentiated by the alkali denaturation technic(1). At birth, 50 to 90% of hemoglobin is of the alkali resistant fetal type, while the remainder is of the normal adult type(2). It is not known, however, whether fetal and adult hemoglobins are distributed evenly inside the fetal red blood cells. In order to study this problem washed erythrocytes of the cord blood were centrifuged and fetal hemoglobin concentrations of the upper and lowest layers were compared.

Material and method. Two samples of blood, 20 cc each, were taken from the cord of 29 normal newborns and added to 0.05 cc of sodium heparin (Abbott, in which 10 mg

TABLE I. Fetal Hemoglobin % Concentration in Cord Blood following Centrifugation.

No.	Non-centrifuged sample	Centrifuged Upper layer	Centrifuged Lowest layer	Difference
1	76	68.8	86.1	17.3
2	75	66	82	16
3	55	49	62	13
4	62	50	73	23
5	58	47	72	25
6	68	58.5	79	20.5
7	80	70	91	21
8	75	66	88	22
9	71	63	81	18
10	89	76	97	21
11	78	69	88	19
12	84	74	91	17
13	85	76	92	16
14	90	79	98	19
15	81	70	91	21
16	74	60	83	23
17	80	70	89	19
18	65	59	83	24
19	78	66	82	16
20	91	80	97	17
21	72	60	84	24
22	81	72	87	15
23	89	80	92	12
24	69	58	79	21
25	87	75	92	17
26	82	71	86	15
27	76	69	89	20
28	85	75	89	14
29	89	77	95	18

= 1000 Toronto units per cc). One of the samples was used to determine the initial value of fetal hemoglobin percent concentration. The washed erythrocytes of the other sample were centrifuged in a graduated tube at 2000 rpm for 30 minutes. Fetal hemoglobin percent concentration was then determined in the upper and lowest layers of the washed erythrocytes with the alkali denaturation technic of Singer *et al.*(1).

Results. The results are shown in Table I. Statistically the differences are highly significant and increase inversely with the lowering of the initial values of fetal hemoglobin percent concentration. The Table shows that centrifugation distributes erythrocytes according to their fetal hemoglobin content. Thus, fetal hemoglobin percent concentration at the bottom of the centrifuged blood is distinctly higher than the initial fetal hemoglobin values. The difference is even more significant when fetal hemoglobin values in the upper and lowest layers are compared.

Comment. This finding may point to the possibility that either fetal and adult hemoglobins are contained in different erythrocytes, or that they are found in various proportions inside the fetal red blood cells.

The assumption that centrifugation of fetal erythrocytes distributes them in layers is also supported by our observation that mean corpuscular volume of the red blood cells at the bottom of the tube is distinctly larger than that of the upper layer.*

The higher concentration of fetal hemoglobin at the bottom of centrifuged blood may be of help in diagnosis of pathological conditions in which fetal hemoglobin blood content is only slightly increased.

Summary. Centrifugation distributes fetal erythrocytes in layers according to their fetal hemoglobin content. The fetal hemoglobin percent concentration is distinctly higher in the bottom layer of centrifuged blood.

* This finding will be reported in detail elsewhere.

1. Singer, K., Chernoff, A. I., and Singer, L., *Blood,* 1951, v6, 413.

2. Singer, K., *Yearbook of Pathology and Clinical Pathology,* Chicago, Yearbook Publishers, 1953-1954.

Received April 26, 1956. P.S.E.B.M., 1956, v92.

A Nutritional Requirement for Bromine. (22431)

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It has been shown by Bosshardt and Huff (1) that the incorporation of an iodinated casein with thyroid activity (Protamone)* into a low-fat diet caused a growth retardation of mice. This depression of growth rate could be prevented partially by feeding fat and completely prevented by feeding cottonseed meal.†

The present study was undertaken to elucidate further the nature of the factor, or factors, in natural materials required by hyperthyroid mice.

Methods. The basal rations employed in these studies had the composition shown in Table I. All substitutions were made at the expense of equal weights of white dextrin. The various diets were fed *ad libitum* to groups of 20-day-old weanling male albino mice obtained from the Sharp and Dohme colony. All studies were of 12 day duration except for one study that was carried for 25 days. In any given experiment groups of 16 mice were employed. The extent of replication of a group is evident from the Table where total number of mice is given.

Results. Diet A (Table I) supported excellent growth in mice. However, when Protamone and Succinylsulfathiazole (SST) were added to this diet a growth retardation resulted. It is to be noted that Diet B, containing Protamone and SST, also contained 0.5% ribose nucleic acid. The ribose nucleic acid was added since it has been shown(2) that under certain dietary conditions an SST-induced growth inhibition in mice could be prevented partially by feeding ribonucleic acid or certain purines or purine derivatives.

It was found that if whey was added to Diet B, a partial reversal of the Protamone-SST induced growth retardation was obtained. The results shown in Table II indicate that the factor(s) in whey responsible for this reversal is water soluble, not adsorbed by Norit or a cation exchange resin (IR 120), and is present in the ash of whey. A dried sea salt preparation, Trace Element Sea Salt (TESS)‡ was found to possess activity similar to that of whey ash (Table II). Since this product was readily obtainable and a very complete mineral analysis was available, it was used as a reference substance for further study.

Chemical studies on TESS that are summarized in Table III show that the activity was adsorbed from water by an anion exchange resin (Dowex-1) and was not adsorbed by a cation exchange resin (IR 120), suggesting that the active material was either an anion or a cation capable of forming an anion. An analysis of TESS, obtained from the Trace Elements Corp., showed the presence of 39 elements. From this list were eliminated those elements that were present in the basal diet, those cations that could not form anions and those anions or anion formers present in quantities so small as not to be expected to stimulate growth under the test conditions. There remained the following 5 elements: aluminum, boron, bromine, fluorine, and rubidium. A mixture of salts of these 5 elements was prepared and fed to mice receiving Diet B in an amount equivalent to that supplied by the addition of 1% TESS to the diet. As shown in Table III this mixture was effective in reversing the Protamone-SST induced growth retardation. The components of this mixture were fed individually in amounts equivalent to those supplied by the addition of 1% TESS to the basal diet. No effect was obtained with the salts of aluminum, boron, fluorine, or rubidium. However, a complete reversal of the Protamone-SST induced growth retardation was obtained with bromine supplied as sodium bromide. Although most studies were of 12 days duration the effects of TESS and bromine were also evident in studies carried for 25 days.

Since it was improbable that a deficiency of a mineral could be induced by a bacterio-

* Cerophyll Laboratories, Inc., Kansas City, Mo.

† Proflo, Traders Oil Mill Co., Fort Worth, Texas.

‡ Trace Elements Corp., Houston, Tex.

TABLE I. Composition of Basal Diets.

	A	B	C
Hydrogenated cottonseed oil*	10.0	10.0	—
Linseed oil	1.5	1.5	—
Hydrogenated coconut oil (Hydrol)†	—	—	10.0
Soybean protein‡	25.0	25.0	25.0
DL Methionine	.3	.3	.3
White dextrin	56.6	50.1	56.1
Salt mix (Jones & Foster) (14)	4.0	4.0	4.0
Cellulose§	2.0	2.0	2.0
Choline methionine tartrate	.6	.6	.6
Iodinated casein (Protamone)	—	4.0	2.0
Succinylsulfathiazole	—	2.0	—
Ribose nucleic acid	—	.5	—

All diets supplemented to contain, per 100 g, 4 mg of α-tocopherol, 900 U.S.P. units vit. A, 180 units vit. D, 1 mg menadione, 0.8 mg thiamine hydrochloride, 1.6 mg riboflavin, 0.8 mg pyridoxine hydrochloride, 4 mg niacin, 4.4 mg calcium pantothenate, 4 mg para-aminobenzoic acid, 20 mg inositol, 0.2 mg folacin, 0.02 mg biotin, and 0.03 mg vit. B_{12}.

* Primex, Procter and Gamble, Cincinnati, O.
† Hydrol, Durkee Famous Foods, Chicago, Ill.
‡ Drackett Assay Protein C-1, Drackett Products Co., Cincinnati, O.
§ Celluflour, Chicago Dietetic Co., Chicago, Ill.

static agent (SST) a study was made to determine the effect of bromine on the growth retardation produced by feeding a diet containing Protamone as the sole deficiency-inducing agent. The results in Table IV show that when a diet free of essential fatty acids and containing Protamone (Diet C) is fed to mice a growth retardation results. This depression of growth rate was prevented partially by feeding linseed oil as a source of linoleic and linolenic acids or by feeding bromine. However, a complete reversal resulted only when bromine and linseed oil were fed in combination. The effect of the unsaturated fatty acids and bromine appears to be additive.

TABLE II. Effect of Whey and Whey Fractions on Growth of Mice Receiving Diets Containing Protamone and Succinylsulfathiazole.

Basal diet	Supplement	Avg gain in wt, 12 day period (g)
A		13.9 (66)
B		8.6 (181)
B	10% Delactosed Whey (DW)*	10.4 (61)
B	H_2O ext. of DW ≏ 10% DW	11.0 (29)
B	Norit filtrate of H_2O ext. of DW ≏ 10% DW	12.4 (198)
B	Ash of Norit filtrate of DW ≏ 10% DW	11.8 (16)
B	IR 120 H filt. of Norit filt. of DW ≏ 10% DW	13.1 (16)
B	1% Trace Element Sea Salt	11.9 (75)

* Consolidated Products Co., Danville, Ill.
Figures in parentheses indicate No. of surviving mice.
Growth increments obtained with all supplements are statistically significant at $P = 0.05$ or less.

Discussion. The results that have been obtained in this study suggest that bromine may

TABLE III. Effect of "Trace Element Sea Salt" and Some of Its Components on Growth of Mice Receiving Diets Containing Protamone and Succinylsulfathiazole.

Basal diet	Supplement	Avg gain in wt, 12 day period (g)	Avg gain in wt, 25 day period (g)
A		13.8 (32)	18.4 (16)
B		10.0 (78)	14.6 (31)
B	.25% Trace Elements Sea Salt (TESS)	12.1 (15)	16.1 (15)
B	.5% TESS	12.5 (16)	17.5 (16)
B	1.0% "	12.1 (32)	16.3 (16)
B	2.0% "	12.8 (16)	16.2 (16)
B	Dowex-1 filt. of TESS ≏ 1% TESS	10.2 (32)	
B	IR 120 filt. " " " " "	11.8 (32)	
B	Mix of Al, B, Br, F, and Rb* ≏ 1% TESS	13.4 (32)	
B	B ≏ 1% TESS	10.0 (16)	
B	Al *Idem*	9.5 (16)	
B	Rb "	9.9 (16)	
B	F "	9.6 (16)	
B	Br ≏ 0.25% TESS, 3.75 ppm.†	12.9 (16)	18.3 (16)
B	Br ≏ 0.5% " 7.5 "	12.6 (32)	16.8 (16)
B	Br ≏ 1.0% " 15 "	13.0 (32)	16.6 (16)
B	Br ≏ 2.0% " 30 "	14.1 (16)	18.9 (16)

* Fed as $Al_2(SO_4)_3 \cdot 18\ H_2O$, $Na_2B_4O_7 \cdot 10\ H_2O$, NaBr, NaF, and RbCl.
† μg of Br/g of diet.
Figures in parentheses indicate No. of surviving mice.

TABLE IV. Effect of Bromine and Linseed Oil on Growth of Protamone-Fed Mice.

Basal diet	Supplement	Avg gain in wt, 12 day period (g)
A		14.9 (15)
C		9.0 (15)
C	15 ppm bromine as NaBr	11.6 (15)
C	1.5% linseed oil	11.5 (15)
C	1.5% linseed oil + 15 ppm bromine	14.5 (16)

Least significant difference for P = .05 = 1.7 g.
Idem P = .01 = 2.2 g.

Figures in parentheses indicate No. of surviving mice.

be a dietary requirement for mice. Bromine has never been implicated as a dietary essential for any animal. Winnek and Smith(3) studied this element a number of years ago and concluded that it was not required by the rat. Bromine is widespread in nature and although few analyses are available it is to be expected that it would be present as a contaminant in many ingredients that are commonly used in the so-called "purified" diets. The analyses of Winnek and Smith(4) illustrate this wide occurrence of bromine in food ingredients. These same authors have shown that feeding bromine to rats causes an increase in the bromine level of such tissues as blood, liver, kidney, muscle, spleen, and brain. It is probable that without special dietary precautions animal tissues will contain bromine as a result of maternal transfer to the offspring or due to the ingestion of bromine-containing foods prior to the usual 21 day weaning period that frequently marks the start of an experimental animal on a "purified" dietary regimen. The growth-promoting effects of bromine that were observed in the present study could be obtained only under conditions of a hyperthyroid-induced deficiency. It is conceivable that the effect of bromine under these conditions was due to a specific reversal of the thyrotoxic activity of the iodinated casein. In view of the small dietary addition providing a response (3.75 ppm) this does not appear to be a logical conclusion.

Abelin and Poretti(5) have shown that in hyperthyroid rats there is an increased uptake of bromine by the liver, kidney, adrenals, brain, and blood. In the present study the effect of hyperthyroidism is interpreted as inducing a partial deficiency of bromine by increasing tissue requirements. The final establishment of a bromine requirement for the mouse probably will have to depend upon the use of weanling mice that have been exposed to a very low bromine intake during their growth period both *in utero* and post-partum up to the time of weaning. The serious difficulties involved in establishing an uncomplicated requirement for this element are reminiscent of the unusually stringent experimental design that was employed by McClendon and Gershon-Cohen when a fluorine requirement for rats was established recently(6).

In those experiments in which a thyroid active material was incorporated into a diet free of essential fatty acids the partial reversal of growth inhibition by linseed oil confirms the experiments of Greenberg(7). The growth stimulation was due undoubtedly to linoleic and linolenic acids, as was shown earlier by these investigators. The effect of bromine was independent of and additive to the fatty acid effect and is similar in certain respects to the "anti-stress" responses of liver fractions that have been reported by Ershoff (8).

An unknown trace element requirement for chicks has been studied recently by several investigators(9-12). Bromine was mentioned specifically in the study of Morrison *et al.* (12) as inactive in supplying the unknown requirement for chicks. A report from this laboratory(13) concerned with the effect of bromine on chick growth deals with this question in more detail.

Summary. Addition of an iodinated casein with thyroid activity to a purified diet adequate in all known essential nutrients produced a growth retardation in mice. Growth inhibition was reversed by fractions of whey and by whey ash. The active inorganic component was found to be bromine.

1. Bosshardt, D. K., and Huff, J. W., *J. Nutrition,* 1953, v50, 117.

2. Huff, J. W., and Bosshardt, D. K., *J. Am. Chem. Soc.,* 1952, v74, 4472.

3. Winnek, P. S., and Smith, A. H., *J. Biol. Chem.,* 1937, v121, 345.

4. Winnek, P. S., and Smith, A. H., *J. Biol. Chem.*, 1937, v119, 93.

5. Abelin, I., and Poretti, G., *Helv. Physiol. et Pharmacol.*, 1952, Acta 10, C7-9.

6. McClendon, J. F., and Gershon-Cohen, J., *J. Agr. and Food Chem.*, 1953, v1, 464.

7. Greenberg, S. M., *J. Nutrition*, 1952, v47, 31.

8. Ershoff, B. H., PROC. SOC. EXP. BIOL. AND MED., 1949, v71, 209.

9. Norris, L. C., *Proc. Dist. Feed Conf.*, 1955, v10, 39.

10. Couch, J. R., Reid, B. L., Camp, A. A., Dannenburg, W. N., and Rozacky, E. E., *ibid.*, 1955, v10, 47.

11. Edwards, H. M., Jr., Chin, D., Norris, L. C., and Heuser, G. F., *J. Nutrition*, 1955, v57, 329.

12. Morrison, A. B., Scott, M. L., and Norris, L. C., *Poultry Science*, 1955, v34, 738.

13. Bosshardt, D. K., Huff, J. W., and Barnes, R. H., PROC. SOC. EXP. BIOL. AND MED., in press.

14. Jones, J. H., and Foster, C., *J. Nutrition*, 1942, v24, 245.

Received April 26, 1956. P.S.E.B.M., 1956, v92

Effect of Bromine on Chick Growth. (22432)

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Huff *et al.*(1) have shown that under certain dietary conditions a hyperthyroid-induced growth retardation in mice could be prevented by feeding selected whey fractions including whey ash or a dried sea salt preparation, Trace Element Sea Salt (TESS).* The active inorganic ingredient was found to be bromine. In view of the interest centered around whey as a source of unidentified growth factors for chicks, the present study was undertaken to determine the effect of bromine on chick growth.

Methods. In 2 of the experiments (1 and 2) one-day-old cockerels (New Hampshire Silver Cornish Cross)† were employed. In experiment 3 one-day-old Nichols strain New Hampshire‡ cockerels and in experiment 4 an unidentified strain of New Hampshire cockerels§ were used. They were housed, in groups of 15, on wire-mesh floors in electrically heated battery brooders. The brooders were lighted continuously. All studies were carried for 31 days. Feed and water were supplied *ad libitum*. All chicks were identified with numbered wing bands and weighed individually at the start of the experiment and weekly thereafter.

* Trace Elements Corp., Houston, Texas.

† Shaw Hatchery, West Chester, Pa.

‡ Moyer Hatchery, Quakertown, Pa.

§ Price Hatchery, Souderton, Pa.

The basal diet, semi-synthetic in nature, employed in these studies had the composition shown in Table I. Whey and TESS substitutions were made at the expense of equal weights of glucose. The sodium bromide supplement was prepared by lyophilizing a water solution with dextrin to give a final concentration of 1.5 mg/g dry weight and was added at the expense of white dextrin in the basal diet.

The results obtained in Exp. 1, 2, and 3 (Table II) showed significant growth responses with whey and bromine. In Exp. 1, a positive effect was obtained with 1% TESS. The addition of 1% TESS to the diet supplied 15 ppm. bromine.

In Exp. 4 no significant growth response was obtained with either whey or bromine.

Discussion. A number of investigators [Norris(2), Couch *et al.*(3), and Edwards *et al.*(4)] have shown that chick growth can be stimulated by a mineral supplement in the form of an ash of natural products such as liver, whey, and distillers solubles. It appears possible from the results obtained in this study that bromine may be an active inorganic material. This suggestion is supported by the work of Huff *et al.*(1) who showed that bromine replaced whey ash or sea salt in the hyperthyroid mouse. On the other hand Morrison *et al.*(5) have reported

TABLE I. Composition of Basal Diet.

G.B.I. vitamin test casein*	18.0
Gelatin	10.0
Glycine	2.0
L Arginine	.5
DL Methionine	.3
Soybean oil	4.0
Glucose†	48.0
White dextrin	3.0
Salt mixture (Jones and Foster (8	6.0
Calcium gluconate	2.5
Celluflour‡	5.0
Choline methionine tartrate	.6

All diets supplemented to contain, per 100 g, 2 mg α-tocopherol acetate, 4 mg menadione, 1100 U.S.P. units vit. A, 250 U.S.P. units vit. D, 200 mg butylated hydroxyanisole,§ 2 mg thiamine hydrochloride, 2 mg riboflavin, 4 mg calcium pantothenate, 10 mg niacin, 2 mg pyridoxine hydrochloride, 0.04 mg biotin, 0.4 mg folacin, 0.01 mg vit. B_{12}, 100 mg inositol, and 30 mg para-aminobenzoic acid.

* General Biochemicals, Inc., Chagrin Falls, O.
† Cerelose, Corn Products Refining Co., Argo, Ill.
‡ Chicago Dietetic Supply Co., Chicago, Ill.
§ Tenox BHA, Tennessee Eastman Co., Kingsport, Tenn.

that several elements including bromine were not responsible for the growth stimulation in chicks that was due to an unknown mineral substance.

In view of the fact that bromine is so widespread in nature and therefore its contamination of dietary ingredients would introduce a serious complication in any type of nutritional study, it is to be expected that reproducibility from experiment to experiment would be difficult. As shown in Exp. 4 of the current study negative results might not be conclusive in the consideration of any specific element unless in that particular study it could be shown that the ash of a natural product or the natural product itself gave a positive growth stimulation. The failure to obtain a growth response from natural products or preparations thereof may indicate a sufficient carry-over from mother to progeny to satisfy the requirements in the early stages of growth. This was recognized by numerous workers when studying vit. B_{12}. More recently it has been shown by Waibel *et al.*(6) that the progeny of hens fed a semi-synthetic or a corn-soybean meal diet were depleted of unidentified growth factors to a greater extent than were the progeny of hens fed a natural diet. Their source of unidentified growth factors was a mixture of dried liver, fish solubles, whey, grass juice, and distillers dried solubles. Winnek and Smith(7) in their reported bromine analyses of a number of natural products found egg albumin to contain a relatively high content of bromine (94 ppm.). This indicates a possible carry-over from the egg to the chick and quite likely is governed primarily by the bromine intake of the hen.

The growth responses to bromine that are noted here are relatively small (approximately 8-10%). Even though they were statistically significant in the three experiments in which positive growth was observed, it is to be expected that negative results will be encountered. It remains for extensive repetition of these studies in nutrition laboratories employing chicks and dietary ingredients derived from different geographical areas to establish the significance of the bromine effect in those areas.

Summary. A growth response to trace addition of bromine to a semi-synthetic diet has been observed in chicks. The conditions required in order to obtain this effect are dis-

TABLE II. Effect of Whey, Sea Salt, and Bromine on Growth of Chicks.

Supplement	Exp. 1 NH × SC	Exp. 2 NH × SC	Exp. 3 NH (Nichols)	Exp. 4 NH
	Avg wt gain, 31 days (g)			
None	449 (15)	448 (29)	480 (30)	458 (30)
5% delactosed whey‡	525†(15)	—	—	—
10% " "	537†(13)	494†(25)	557†(26)	480 (29)
8 ppm Br as NaBr	487*(14)	—	—	458 (29)
15 " " " "	489*(14)	482†(26)	535†(29)	—
1% TESS	496†(12)	—	—	—

* Indicates significance $P < .05$.
† " " $P < .01$.
‡ Consolidated Products Co., Danville, Ill.
Figures in parentheses indicate No. of chicks surviving.

cussed.

1. Huff, J. W., Bosshardt, D. K., Miller, O. P., and Barnes, R. H., Proc. Soc. Exp. Biol. and Med., in press.

2. Norris, L. C., *Proc. Dist. Feed Conf.*, 1955, v10, 39.

3. Couch, J. R., Reid, B. L., Camp, A. A., Dannenburg, W. N., and Rozacky, E. E., *ibid.*, 1955, v10, 47.

4. Edwards, H. M., Jr., Chin, D., Norris, L. C., and Heuser, G. F., *J. Nutrition*, 1955, v57, 329.

5. Morrison, A. B., Scott, M. L., and Norris, L. C., *Poultry Sci.*, 1955, v34, 738.

6. Waibel, P. E., Morrison, A. B., and Norris, L. C., *ibid.*, 1954, v33, 1085.

7. Winnek, P. S., and Smith, A. H., *J. Biol. Chem.*, 1937, v119, 93.

8. Jones, J. H., and Foster, C., *J. Nutrition*, 1942, v24, 245.

Received April 26, 1956. P.S.E.B.M., 1956, v92.

Influence of Testis on Induction of Ovarian Tumors of Mice by X-rays. (22433)

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Absence of ovarian tumorigenesis when only a single ovary is irradiated suggests that the mechanism of induction of mouse ovarian tumors by x-rays is similar to that involved in tumor development within an ovary transplanted to a castrated animal's spleen(1). In either case a functional ovary *in situ* inhibits the development of ovarian tumors; this inhibition is said to result from suppression of output of excessive pituitary gonadotrophin (1,2).

Results. When Balb/c female mice received 200r of x-rays, ovarian tumors were present 16 months post-irradiation in 20 of 22. Ovarian tumor development was suppressed in all of 36 mice by isologous Balb/c subcutaneous ovarian grafts made within 24 hours following irradiation. If only a single ovary was irradiated, tumors developed in 3 of 37, indicating suppression of tumorigenesis by the functional ovary in 92%. When the functional ovary was removed immediately post-irradiation, tumors appeared in 58 of 66 irradiated ovaries. Only those ovaries exceeding 3 mm in diameter were considered tumorous.

Injection of estrogenic hormone has been shown to inhibit x-ray induced ovarian tumorigenesis, whereas injected androgen was relatively ineffective(3). To determine whether the functioning testis inhibits the development of ovarian tumors by x-rays, x-irradiated ovaries were grafted subcutaneously into both intact and castrated male Balb/c mice; the donor Balb/c females and the recipient males of this strain were 6-8 weeks of age at the time of grafting. Grafts were made within an hour after whole body x-irradiation of the intact female donor. The x-ray-treated mice were exposed to general body irradiation in cardboard boxes at 30 cm target-skin distance, 140 kv, 2-1 mm aluminum filters, dose rate 70r per minute.

Ovarian tumors developed in 10 of 12 intact males bearing irradiated ovarian transplants (13 of 24, or 54% of grafts). Similar tumors appeared in irradiated ovaries residing in all of 18 castrated male hosts (29 of 34, or 85% of grafts). The latent period of tumor induction was not significantly different in castrated and intact mice (12-16 months). Except for a tendency toward areas of undifferentiated carcinoma, histologically the neoplasms were the same as those occurring in the Balb/c strain following irradiation of the ovary *in situ*(1). Although seminal vesicle development was not maximum in all intact graft-bearing males, stimulation was sufficient to indicate testicular androgenic activity.

* This investigation was supported by grant from National Cancer Institute, P.H.S.

In contrast to ovarian secretion, which remarkably inhibits x-ray induced ovarian tumorigenesis in mice, secretion of the testis is not similarly inhibitory. Presently Balb/c ovaries are being grafted into the spleens of intact and castrated Balb/c mice to determine the degree to which ovarian tumor development induced by transplanting an ovary into the spleen is inhibited by the testis. Adequate data on this point do not appear in the literature.

Testosterone inhibits the abnormal release of pituitary gonadotrophin after x-irradiation of female mice(4). Since neither injected (3) nor endogenously produced androgen effectively (in contrast to estrogen) inhibits ovarian tumorigenesis, in many cases exposure of the ovaries to elevated blood levels of gonadotrophins may not be essential for ovarian tumor formation induced by x-rays. It is possible that estrogenic hormone may act directly on the ovary to suppress tumor development.

Summary. When irradiated ovaries were grafted into intact as well as castrated male Balb/c mice, ovarian tumorigenesis proceeded. Functional normal ovarian grafts into previously irradiated Balb/c females inhibited tumorigenesis in the irradiated ovary. It appears that irradiation-induced ovarian tumorigenesis in this inbred strain is inhibited almost completely by ovarian, but only to a minor degree by testicular secretion.

1. Lick, L., Kirschbaum, A., and Mixer, H. W., *Cancer Research,* 1949, v9, 532.

2. Li, M. H., and Gardner, W. U., *ibid.,* 1947, v7, 549.

3. Gardner, W. U., PROC. SOC. EXP. BIOL. AND MED., 1950, v75, 434.

4. Vermande-Van Eck, G., and Chang, C. H., *Cancer Research,* 1955, v15, 280.

Received April 27, 1956. P.S.E.B.M., 1956, v92.

Presence of Erythropoietic Factor in Plasma of Normal and Hypophysectomized Rats Following Bleeding.* (22434)

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Carnat and Deflandre(1) discovered a factor in the plasma of anemic rabbits which would stimulate erythropoiesis. This factor, "erythropoietine" or "hematopoietine," has been demonstrated by many other investigators in the plasma of rabbits made anemic by repeated bleeding or by injection of phenylhydrazine(2-8). It has also been demonstrated in the plasma of rats(9,10) and in the human(11).

The influence of the endocrine glands on hemopoiesis is being actively investigated, and the anemia which follows removal of the hypophysis is well established; the present investigation was undertaken in an attempt to determine whether the anemia in the hypophysectomized animal can be related to the absence of such a plasma factor.

Methods. Adult female rats of 3 to 4 months of age and of the Sprague-Dawley strain were used in this experiment. All rats were fed Purina chow *ad libitum* supplemented once a week by lettuce. Completeness of hypophysectomies was verified by examination of the organ site at autopsy with a binocular dissecting microscope. *Donor rats,* from which plasma was obtained, were divided into 6 groups: (1) normal unbled rats; (2) and (3) bled normal rats; (4) hypophysectomized rats not yet anemic; and (5) and (6) bled hypophysectomized animals. One group of normal rats was made anemic by removing 4 cc of blood on day 1, and 2 cc on day 3, the rats being exsanguinated on day 5. At that time the hematocrit averaged 22% as compared to 43% for the normal unbled

* This investigation was supported by a research grant (A476) from National Institute of Arthritis and Metabolic Diseases of N.I.H., P.H.S.

† With technical assistance of Mrs. Barbara Giffen.

rats. A second group of normal rats was bled 4 cc on day 1, 2 cc on day 3, and either 2 or 3 cc on day 5. Blood was obtained on the 6th day. In spite of the fact more blood was removed from these rats than from the first group, the hematocrits averaged 25%. In the first group of bled hypophysectomized rats, 3 cc of blood were removed on day 1 only and the animals exsanguinated on day 5. The hematocrits averaged 27% compared to 41% for the unbled hypophysectomized rats. A second group of hypophysectomized rats was exposed to a more severe hemorrhage, 3 cc of blood being removed on day 1, and 2 additional cc on day 3. These animals were exsanguinated on day 5, the hematocrits averaging 21%. *Blood* from each group of rats was placed in tubes containing heparin and centrifuged. The plasma so obtained was injected into normal rats (average body weight = 193 g) in either 2 cc or 3 cc doses per day for 3 consecutive days, injections being given intravenously via tail veins. Reticulocyte counts were used as an index of erythropoietic stimulation. Blood for this study was obtained by heart puncture 24 and 48 hours after the last injection. Reticulocytes were stained with New Methylene Blue according to the method of Brecher(12).

Results. The results obtained are presented in Fig. 1. Plasma from unbled normal rats (6.0 cc total) did not produce a reticulocyte response when injected into normal recipients. The same amount of plasma from donor rats which were bled to such an extent that the hematocrit averaged 22% (normal—43%) did induce a response, the reticulocyte count 24 and 48 hours after the last injection being 1.6%, an increase of 60%. As these figures were not statistically significant, the experiment was repeated using a total dose of 9.0 cc of plasma rather than 6.0 cc. Under these circumstances the reticulocyte response was highly significant, reaching levels 367 and 483% above normal 24 and 48 hours after the last injection respectively.

When rats which had been hypophysectomized 40 days previously but which exhibited a nearly normal hematocrit of 41% served as donors (6.0 cc total), the reticulocyte

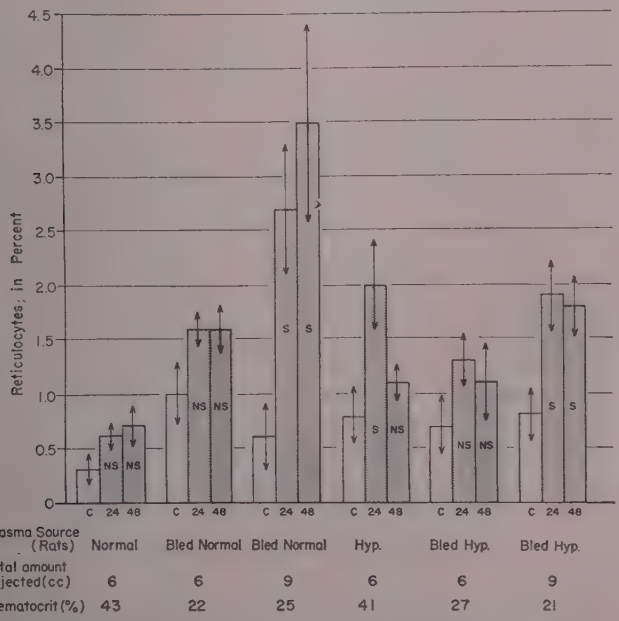

FIG. 1. Reticulocyte response to injections of 6 or 9 cc of plasma obtained from normal rats, bled normal rats, hypophysectomized rats, and bled hypophysectomized rats. Each value is an avg of counts on 5 or 6 animals. C = Control values (before inj.); 24 = 24 hr after last inj.; 48 = 48 hr after last inj. Vertical arrows = stand. error; S = significantly (Fisher's "*t*") different than controls; NS = not significantly different than controls.

counts were elevated in the normal recipients to 150% above normal 24 hours after the last injection. However, the number of reticulocytes had returned to normal 48 hours after injections had ceased. 6 cc of plasma from hypophysectomized rats bled to such a degree that the hematocrit reading was 27% induced increases in reticulocyte percentages in the normal recipient rats (86% above normal at 24 hours and 57% at 48 hours) which were not statistically significant. Accordingly, hypophysectomized rats were bled more severely until the hematocrit reading averaged 21% and the amount of plasma injected into normal recipient rats was increased from a total of 6 cc to 9 cc. Under these circumstances the reticulocyte percentage was significantly increased to 1.9% at 24 hours and 1.8% at 48 hours after the last injection, the 2 figures being 138% and 125% above those obtained before injections started (0.8%).

Discussion. These data support the work of others(9,10) that a plasma erythropoietic factor can be exhibited in rats, as well as rabbits, made anemic by bleeding. In addition, they also suggest that a lack of this factor is not playing a role in post-hypophysectomy anemia for it can be formed in the absence of the hypophysis.

The results obtained with plasma from unbled hypophysectomized rats are difficult to explain. There is a possibility that such an animal might be elaborating an erythropoietic factor in response to a decreased erythropoiesis in spite of the fact that the hematocrits indicated that the animals were not as yet anemic. A more thorough study of this aspect of the problem is in progress. Another point which should be noted is the relatively lesser response to plasma from bled hypophysectomized animals as compared to the response to plasma from bled normal rats.

Summary. 1. Normal adult-female rats were injected intravenously with a total of 6 or 9 cc of plasma, over a 3-day period, obtained from normal rats, bled normal rats, hypophysectomized rats, or bled hypophysectomized rats. Reticulocyte response was used as criterion of erythropoietic activity and blood was removed for reticulocyte counts 24 and 48 hours after the last plasma injection. 2. Best results were obtained with the larger dose level. Erythropoietic factor was present in the plasma of the bled normal rats, the reticulocyte count being elevated 483% above normal. The plasma factor was also found to be present in bled hypophysectomized rats, the reticulocyte count of the recipient rats being elevated 138% above normal levels. This factor was also found in the plasma of unbled hypophysectomized rats, a fact which needs further investigation.

Conclusions. This experiment indicates that erythropoietic factor can be demonstrated in normal bled rats and that hypophysectomy does not prevent its formation.

1. Carnat, P., and Deflandre, C., *Compt. rend. Acad. sc.,* 1906, v143, 384.

2. Gibelli, C., *Arch. f. Exp. Path. u Pharmakol,* 1911, v65, 248.

3. Forster, J., and Kiss, F., *Biochem. Z.,* 1925, v160, 442.

4. Borsook, H., Deasy, C. L., Haagen-Smit, A. J., Keighley, G., and Lowy, P. H., *J. Biol. Chem.,* 1952, v196, 669.

5. Erslev, A., *Blood,* 1953, v8, 349.

6. Borsook, H., Graybiel, A., Keighley, G., and Windsor, E., *ibid.,* 1954, v9, 734.

7. Gordon, A. S., Piliero, S. J., Kleinberg, W., and Freedman, H., PROC. SOC. EXP. BIOL. AND MED., 1954, v86, 255.

8. Hodgson, G., and Tohá, J., *Blood,* 1954, v9, 299.

9. Plzak, L. F., Fried, W., Jacobson, L. O., and Bethard, W. F., *J. Lab. and Clin. Med.,* 1955, v46, 671.

10. Stohlman, F., Jr., and Brecher, G., PROC. SOC. EXP. BIOL. AND MED., 1956, v91, 1.

11. Stohlman, F., Jr., Rath, C. E., and Rose, J. C., *Blood,* 1954, v9, 721.

12. Brecher, G., *Am. J. Clin. Path.,* 1949, v19, 895.

Received April 30, 1956. P.S.E.B.M., 1956, v92.

Effect of Ganglionic Blocking Agents on Pressor Responses Induced by Splanchnic Faradization. (22435)

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The efferent components of the greater splanchnic nerve of mammals are generally considered to be largely preganglionic with only a small postganglionic representation (1-5). Synapses of these preganglionic fibers occur primarily in the celiac and aorticorenal ganglia except for those fibres which go directly to the adrenal medullae, this innervation being considered a modified "ganglionic" junction. Stimulation of the peripheral end of the divided splanchnic nerve leads to a sympatho-adrenal discharge which is associated with an arterial pressor response. It might be anticipated that ganglionic blocking agents would markedly reduce this pressor response.

Recently reports(6,7) have appeared which indicate that ganglionic blocking agents have effects on the visceral circulation of the dog which are inconsistent with the decrease in peripheral resistance and increase in flow which would be expected following interruption of sympathetic vasomotor tone. The following study was carried out to determine some of the effects of ganglionic blocking agents on visceral circulation.

Materials and methods. A total of 25 cats of both sexes, anesthetized with Dial-urethane, were used in this study. The left splanchnic nerve was exposed through a lateral incision in the abdominal wall and transected near its exit from the diaphragm. Operative procedures generally resulted in a 20 mm Hg drop in arterial pressure. A platinum electrode was placed on the peripheral end of this nerve and stimulation applied with an Electrodyne stimulator at 5 v, 100 u sec duration, at 30 per second for 15 seconds. The carotid pressure was recorded with an Anderson glass capsule manometer(8). All drugs were administered intravenously from 5 to 15 minutes prior to stimulation, depending upon their duration of action. Stimulations were spaced 30 to 45 minutes apart. Each stimulation was followed by an injection of 70% epinephrine—30% norepinephrine (epi + n-epi) mixture. According to Mirkin and Bonnycastle(9), this approximates adrenal effluent proportions following splanchnic stimulation. Doses (2-6 μg/kg) were selected to give a pressor response equivalent to that obtained by the control splanchnic stimulation.

Results. Effect of ganglionic blocking agents on pressor responses induced by left splanchnic nerve faradization. Four ganglionic blocking agents were tested for their capacity to antagonize the pressor response due to splanchnic stimulation: tetraethylammonium bromide (TEA), hexamethonium chloride, pentapyrrolidinium bitartrate and chlorisondamine chloride (Ecolid™, Ciba). TEA, hexamethonium and pentapyrrolidinium yielded essentially the same results. In two animals for each compound, doses of 1, 5 and 10 mg/kg failed to obliterate or antagonize the pressor response; on the contrary, they provided a clear-cut augmentation of the response to stimulation and to injected epi + n-epi (Fig. 1). Chlorisondamine chloride in doses of 320 μg, and 1, 5 and 10 mg/kg, similarly failed to antagonize the response to stimulation but did not augment it, nor did this compound potentiate the response to injected epi + n-epi.

Interpretation of the results is complicated by the presence of the left adrenal gland in this series of experiments. For, while ganglionic blockade may have reduced the neural vasoconstrictor components of the pressor response, the release of epinephrine and norepinephrine from the adrenal medulla may not have been blocked, and under the simulated denervation of ganglionic blockade may have given rise to a markedly enhanced pressor effect. To eliminate this possibility, another group of cats was run in which the left

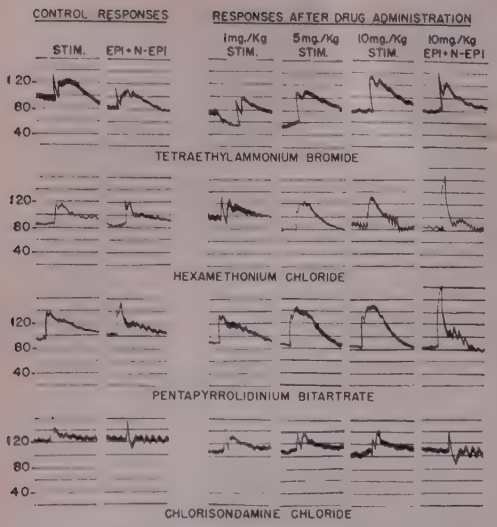

FIG. 1. Arterial pressor responses elicited by left splanchnic nerve faradization in presence of ganglionic blocking agents.

splanchnic was stimulated following removal of the ipsilateral adrenal gland.

Effect of ganglionic blocking agents on pressor response induced by left splanchnic nerve faradization with left adrenal ablated. The procedure outlined above was followed except that the left adrenal was removed. In 2 animals for each compound, TEA, pentapyrrolidinium and chlorisondamine chloride in doses of 1 to 10 mg/kg did not block or antagonize the pressor response concomitant with splanchnic stimulation, but, on the contrary, augmented it. Hexamethonium neither antagonized nor augmented the response to stimulation. All four compounds potentiated the response to injected epi + n-epi (Fig. 2). To substantiate the fact that we were dealing with vasoconstrictor impulses and thus a response mediated peripherally by norepinephrine, 2 mg/kg of the adrenergic blocking agent, phentolamine, was injected intravenously at the end of each experiment and followed by a stimulation and an injection of epi + n-epi. Phentolamine acts to lessen the pressor effect of norepinephrine and reverses the effect of epinephrine to a depressor effect. In these experiments this substance always reduced, but did not reverse, the pressor response to splanchnic nerve stimulation. The pressor effect following the injection of epi + n-epi, however, was reversed.

Discussion. Since the efferent composition of the greater splanchnic nerve is generally taken to be largely preganglionic, the evidence presented in this paper tends to cast doubt upon the capacity of high doses of ganglionic blocking agents to block effectively transmission of vasoconstrictor impulses through certain collateral sympathetic ganglia in the cat. An alternative view is that these ganglia are blocked and that the pressor response induced by splanchnic stimulation is due to a potentiation of the vasoconstrictor activity carried by the numerically small complement of postganglionic efferents. But since complete blockade at the ganglia would produce a very marked reduction in the total number of impulses reaching visceral arterioles, it would be necessary to postulate that ganglionic blockade produces an enormous potentiation of the activity of the neurohumoral substances released by the few postganglionic fibers. The responses to injected epi + n-epi are potentiated only two to three times at most by ganglionic blocking agents and thus make this alternative view seem unlikely.

These findings may well help to explain

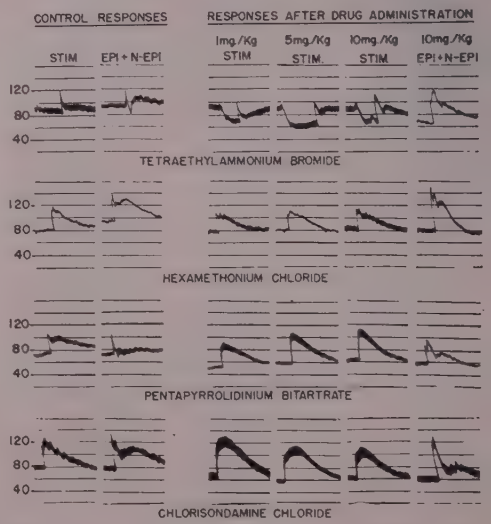

FIG. 2. Arterial pressor responses elicited by left splanchnic nerve faradization in presence of ganglionic blocking agents and with the left adrenal ablated.

the observations in the dog by Plummer *et al.* (6) with chlorisondamine chloride and those of Trapold(7) with chlorisondamine chloride, hexamethonium and pentapyrrolidinium, that peripheral resistance of the mesenteric bed is not greatly decreased after administration of such agents.

That certain sympathetic pathways should not be affected by ganglionic blocking agents is not a novel concept. Freyburger *et al.*(10) and Pardo *et al.*(11) have presented evidence to this effect, and Moe and Freyburger(12) have discussed the subject at length. Cicardo and Dutrey(13) have also presented evidence indicating the ineffectiveness of ganglioplegic agents to block certain ganglionated pressor pathways.

Evidence has been presented by Freyburger *et al.*(14) and Morrison and Farrar(15) that TEA can block the release of epinephrine from the adrenal medulla following splanchnic nerve stimulation. It is not known whether the other blocking agents possess this activity. Apparently, however, epinephrine released from the medulla plays a subsidiary part in the pressor response elicited by faradization of the splanchnic nerve(16).

Summary. The effects of the ganglionic blocking agents, TEA, hexamethonium, pentapyrrolidinium and chlorisondamine chloride on the pressor responses induced by peripheral splanchnic faradization have been determined in 16 cats. These agents did not antagonize the pressor responses when the ipsilateral adrenal gland was present or after it had been ablated. The significance of these findings is discussed.

1. Ranson, S. W., and Billingsley, P. R., *J. Comp. Neurol.,* 1918, v29, 405.

2. Ranson, S. W., and Clark, S. L., *The Anatomy of the Nervous System,* W. B. Saunders, Philadelphia, 1947.

3. Reighard, J., and Jennings, H. S., *Anatomy of the Cat,* Henry Holt and Co., New York, 1935.

4. Mitchell, G. A. G., *Anatomy of the Autonomic Nervous System,* E. & S. Livingstone, Ltd., Edinburgh, 1953.

5. *Gray's Anatomy,* 25th ed., Lea and Febiger, 1948.

6. Plummer, A. J., Trapold, J. H., Schneider, J. A., Maxwell, R. A., and Earl, A. E., *J. Pharm. and Exp. Therap.,* 1955, v115, 172.

7. Trapold, J. H., Abstract— Meetings of the Am. Soc. for Pharm. and Exp. Therap., Sept., 1955.

8. Anderson, F. F., *J. Lab. and Clin. Med.,* 1941, v9, 1520.

9. Mirkin, B. L., and Bonnycastle, D. D., *A. J. P.,* 1954, v178, 529.

10. Freyburger, W. A., Gruhzit, C. C., and Moe, G. K., *ibid.,* 1950, v163, 290.

11. Pardo, E. G., Rennick, B. R., and Moe, G. K., *ibid.,* 1950, v161, 245.

12. Moe, G. K., and Freyburger, W. A., *Pharm. Rev.,* 1950, v2, 61.

13. Cicardo, V. H., and Dutrey, E. D., *Rev. de La Soc. Arg. de Biol.,* 1954, v30, 132.

14. Freyburger, W. A., Gruhzit, C. C., Rennick, B. R., and Moe, G. K., *A. J. P.,* 1950, v163, 554.

15. Morrison, J. L., Farrar, C. H., PROC. SOC. EXP. BIOL. AND MED., 1949, v71, 82.

16. Gley, E., and Quinquand, A., *J. de Physiol. et de Pathol. Gen.,* 1921, v19, 355.

Received April 30, 1956. P.S.E.B.M., 1956, v92.

Effects of Reserpine on Urinary Bladder Tension. (22436)

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The most prominent actions of reserpine are its "tranquilizing" and blood pressure lowering effects(1-3). Both of these responses are slow in onset, generally commencing from one to several hours after intravenous injection. However, slowness of onset is not characteristic of all the pharmacological responses to this compound. The present communication deals with the response of the urinary bladder to reserpine, an action which begins within 5 minutes after administration.

Methods. Twenty female dogs anesthetized with 30 mg/kg intraperitoneally, plus a continuous intravenous infusion of 0.1 mg/kg/

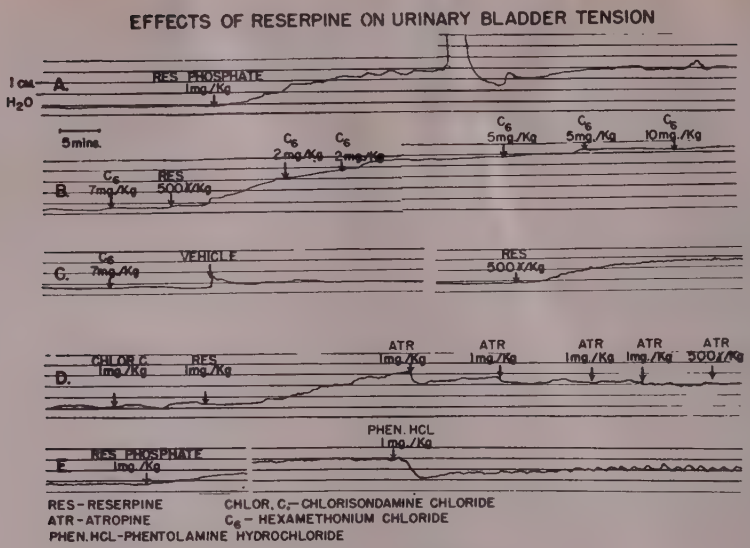

FIG. 1. Reserpine-induced increase in urinary bladder tension and effects of autonomic blocking agents on this response.

minute of pentobarbital were employed in this study. Urinary bladders were exposed through a mid-ventral incision and the ureters ligated and cut. Bladders were then catheterized and connected to a water manometer.

Results. Effects of reserpine on urinary bladder tension. Reserpine administered intravenously as the water-soluble phosphate or in the commercial vehicle* induces an immediate and prolonged increase in urinary bladder tension (Fig. 1-A). One-half to 1 mg/kg will produce a rise of approximately 2 to 4 cm H_2O which lasts over an indefinite period. Generally the bladder is quiescent, but in an occasional animal, during the sustained increase in bladder tension, it may exhibit a transient total contraction (Fig. 1-A). Since the most prominent effects of reserpine are generally attributed to a central action, it was considered of interest to determine the effects of ganglionic blockade on this response.

Effect of ganglionic blockade on response of bladder to reserpine. Intravenous doses of 1 to 7 mg/kg of hexamethonium chloride and 0.3 mg to 1 mg/kg of chlorisondamine chloride administered prior to reserpine were without effect on the bladder response (Fig. 1-B, C, D). High doses of hexamethonium chloride administered after reserpine were also without effect. Administration of 0.5 to 1 mg/kg of reserpine produced the same effects on the bladder as in the absence of these agents. Reserpine vehicle produced no marked effects on bladder tension (Fig. 1-C). Ganglionic blocking agents in themselves did not affect bladder tension.

Effects of cholinergic and adrenergic blocking agents and LSD on bladder response to reserpine. Atropine in intravenous doses of 1-4 mg/kg produced slight to moderate reduction of the increased bladder tension produced by reserpine (Fig. 1-D). The adrenergic blocking agent, phentolamine, was very effective in reducing the increased bladder tension. Doses of 1 mg/kg consistently lowered the bladder tension to control levels for a period of one-half hour, after which bladder tension returned to its previous level (Fig. 1-E). LSD administered after reserpine in intravenous doses of 20-40 μg/kg did not reduce, but on the contrary, markedly increased bladder tension. LSD also produced a marked increase in bladder tension when given alone.

Effect of reserpine on response of urinary bladder to choline esters. Carbamylcholine and mecholyl in intravenous doses ranging

* Ciba vehicle.

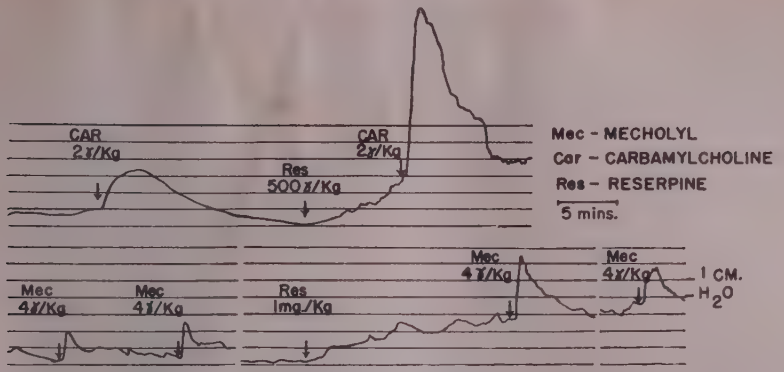

FIG. 2. Effect of reserpine on the response of the urinary bladder to intravenously-administered choline esters.

from 0.1 to 5 μg/kg will produce transient, repeatable contractions of the urinary bladder. After intravenous administration of 0.5 to 2 mg/kg of reserpine, moderate to marked potentiation of the response to these agents could be demonstrated (Fig. 2). In some cases after reserpine administration, these agents produced rapid, transient, total contraction of the bladder.

Discussion. The responses of the central nervous system and blood pressure to reserpine are slow in onset and therefore possibly reflect an action not initiated by the reserpine molecule *per se,* but by some metabolite of it, or to an agent such as serotonin which has been shown to be released from the brain and intestinal tract of rabbits(4,5) by reserpine. Unlike these effects, the onset of the response of the urinary bladder is almost immediate and therefore may be initiated by the unaltered reserpine molecule. That released serotonin may play no important role in this effect is indicated by the fact that the serotonin antagonist LSD does not reduce the increased bladder tension. Unfortunately this evidence is not conclusive, for LSD in itself can produce an increase in bladder tonus which may mask any of its antagonizing effects on this response. However, Shore *et al.* (6) report that in the dog a marked increase in the urinary excretion of 5-hydroxy-indole acetic acid, a major metabolite of serotonin, occurs in the dog following reserpine injection. It is difficult to see how released serotonin, which is rapidly inactivated, could be the cause of the prolonged bladder contraction.

The bladder response to reserpine is apparently a reflection of a peripheral action, since it can occur in the presence of large doses of ganglionic blocking agents. Since atropine is partially active in reducing bladder tension, there is a possibility of a cholinergic-like or cholinergic-potentiating action of reserpine upon the bladder muscle. The fact that reserpine has the capacity to potentiate the response of the bladder to mecholyl and carbamylcholine is supportive evidence for this view.

Since the adrenergic blocking agent, phentolamine, can reduce the increased bladder tension, it would appear that some sympathetic humoral substance may also be involved. It is not inconceivable that reserpine could be acting to produce a prolonged release of epinephrine or norepinephrine from some site, possibly the adrenal medullae.

Summary. Reserpine will produce a gradual increase in bladder tension which is initiated within five minutes after intravenous injection. This response is not blocked by ganglionic blocking agents. Atropine has a slight to moderate antagonizing effect on this response. The adrenergic blocking agent, phentolamine, has a marked antagonizing effect on this response. Reserpine can potentiate the response of the bladder to mecholyl and carbamylcholine. The significance of these facts is discussed.

1. Bein, H. J., *Experientia,* 1953, v9, 107.

2. Plummer, A. J., Earl, A., Schneider, J. A., Trapold, J., and Barrett, W., *Ann. N. Y. Acad. Sci.*, 1954, v59, 8.

3. Trapold, J. H., Plummer, A. J., and Yonkman, F. F., *J. Pharm. and Exp. Therap.*, 1954, v110, 205.

4. Pletscher, A., Shore, P. A., and Brodie, B. B., *ibid.*, 1956, v116, 84.

5. ———, *Science*, 1955, v122, 374.

6. Shore, P. R., Silver, S. L., and Brodie, B. B., *ibid.*, 1955, v122, 284.

Received April 30, 1956. P.S.E.B.M., 1956, v92.

Antiviral Action of Helenine on Experimental Poliomyelitis.* (22437)

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Recently crude preparations from 2 species of penicillium, *P. stoloniferum* and *P. funiculosum* have been reported to be effective against certain small neurotropic viruses such as Semliki Forest, MM, and Columbia SK (1-3). The crude filtrate of *P. stoloniferum*, known at M5-8450(4), was also found to be effective against poliomyelitis virus inoculated by peripheral routes in mice(5) and monkeys (6). In view of the suspected similarity between M5-8450 and Helenine, the antiviral agent from *P. funiculosum*(4,7), it was of interest to investigate the effect of Helenine on experimental poliomyelitis in mice and monkeys.

Materials and methods. Young male and female cynomolgus monkeys, weighing between 1.1 and 2.8 kg, and young Swiss albino mice, weighing 12 g or less, were used in these studies. Mice were inoculated intraperitoneally with approximately 10 ID_{50} of the MEF_1 strain of type II poliomyelitis virus adapted to suckling hamsters by Powell and Culbertson(5), while monkeys were inoculated subcutaneously with an estimated 100 ID_{50} of the Mahoney strain of type I poliomyelitis virus. All animals were observed daily for the onset of paralysis throughout an observation period of at least 21 days for mice, and at least 30 days for monkeys. Helenine† was obtained as a 10-fold concentrate of the acetone-precipitated material. For purposes of comparison, one experiment included a group of monkeys treated with M5-8450.† Both materials were kept frozen at −70°C until the day of use.

Results. In preliminary tests the maximum daily dose of Helenine tolerated by mice was found to be 0.5 ml of 2-fold concentrate (8). No toxic effects other than a transitory anorexia were observed in monkeys receiving up to 5 ml of 10-fold concentrated Helenine per kg twice daily for 4 days. More extensive toxicity studies were not conducted because of the difficulty of determining whether observed effects were due to the active principle or to impurities in the available preparation.

Data showing the effect of Helenine-treatment in mice infected with poliomyelitis virus are given in Table I. Mice were given Helenine daily for 4 days beginning the day before virus inoculation. The first, third and fourth doses were injected intraperitoneally while the second dose, given the day of virus inoculation, was injected subcutaneously. In only one experiment with mice did Helenine appear to reduce the incidence of paralysis, but this difference is not significant at the 5% level when the data are analyzed accord-

*Aided by grant from National Foundation for Infantile Paralysis.

† The authors wish to thank Dr. Laurella McClelland, Merck Institute for Therapeutic Research, for the supply of Helenine and Dr. William S. Boniece, Lilly Research Laboratories, for the M5-8450.

TABLE I. Effect of Helenine on Poliomyelitis in Mice.*

Treatment†	Treated		Control	
	Morbidity	Incubation period	Morbidity	Incubation period
Helenine (2×) .5 ml/day, ×2	11/17	8.5	16/19	5.9
" " .5 " ×4	15/20	7.6	16/19	5.9
" (5×) .1 " ×4	18/19	5.0	17/20	3.1

* Inoculated intraperitoneally with approximately 10 ID_{50}'s of MEF_1 poliomyelitis virus.
† Given intraperitoneally beginning day before virus inoculation except on day of virus inoculation when Helenine was given subcutaneously.

ing to the procedure of Mainland and Murray(9). In all cases, however, there appeared to be some prolongation of the incubation period. Using the harmonic mean survival time of the treated and control groups one may derive a survival index similar to that described by Shope(2). When mice were treated with 0.5 ml of 2x Helenine for 2 days the survival index was 1.44, and the indices calculated for the other experiments with mice were 1.29 and 1.47 respectively.

In monkeys, Helenine was injected intraperitoneally as the 10-fold concentrated acetone-precipitate; 2 doses of 5 ml per kg each were given the day before virus, the third dose 4 hours before and the fourth dose 4 hours after virus inoculation, a schedule similar to that previously employed with M5-8450(6). In the present study M5-8450 was re-constituted with sterile distilled water to one-half its original volume and administered on the same schedule. Results of these tests in monkeys are given in Table II. From these data it is seen that treatment with Helenine was effective in protecting monkeys against infection with poliomyelitis. When the data from the monkey experiments are combined it may be seen that the incidence of paralysis was reduced from 100% (11/11) in the control group to 18% (2/11) in the Helenine-treated group. This difference in morbidity between the treated and control groups is significant at a level of less than .01 using the test previously cited(9). Treatment with Helenine, in addition to reducing the incidence of paralysis, also produced a delay in the onset of the disease. The average time to the onset of paralysis in those Helenine-treated monkeys that eventually developed paralysis was 23.5 days, and in the M5-8450-treated monkeys 30.5 days, while the average time of onset of paralysis in the control monkeys was 8.8 days. Survival indices calculated as described above were 18.5 and 12.2 for the Helenine-treated groups and 5.6 for the group receiving M5-8450.

In view of the marked effectiveness of Helenine as a prophylactic agent, an attempt was made to detect therapeutic activity. Ten monkeys were infected subcutaneously with the Mahoney strain of poliomyelitis virus. Rectal temperatures were recorded daily and when an animal exhibited a temperature of at least 104°F, or showed evidence of paralysis, treatment was initiated. Alternate animals in the order of onset received Helenine. 5 ml of 10x-concentrate per kg twice daily for not more than 4 days, or equal volumes of buffered saline intraperitoneally. The group of 5 monkeys which was treated with Helenine

TABLE II. Effect of Helenine and M5-8450 in Prevention of Poliomyelitis in Cynomolgus Monkeys.*

Treatment	Treated		Control	
	Day of onset	Morbidity	Day of onset	Morbidity
Helenine (10 ×) 5 ml/kg, IP × 4, twice day before, twice day of virus inoc.	27, S, S, S, S, S	1/6	7, 8, 9, 9, 10, 13	6/6
Helenine (10 ×); same	20, S, S, S, S	1/5	7, 7, 8, 10, 10	5/5
M5-8450 (2 ×), 12.5 ml; same	15, 46, S, S, S	2/5	"	"

* Inoculated subcut. with estimated 100 ID_{50} of Mahoney strain of Type I poliomyelitis virus.

and the 5 control animals each had an average survival time of 2.6 days after the detectable onset of disease. These data indicate that impure preparations of Helenine such as were available had marked prophylactic activity against poliomyelitis in monkeys but no apparent therapeutic activity.

Discussion. Helenine, like M5-8450(6), was found to be an effective prophylactic against poliomyelitis in monkeys. In contrast to its effectiveness in monkeys, Helenine did not alter significantly the incidence of paralysis in mice, although it did appear to prolong the incubation period. A species difference in the response of the host to this material seems unlikely in view of the effectiveness of Helenine against other neurotropic viruses in mice(2,3). One can speculate that the observed effects may have represented type specific differences in susceptibility between types I and II poliomyelitis viruses. Type differences have been encountered in susceptibility to inactivation with merthiolate in the 1954 poliomyelitis vaccine(10) and have been reported to occur *in vitro* with certain chemical agents(11). On the other hand, differences in effectiveness also could have been due to differences in reactivity of the whole host-virus complex; Mahoney virus in the monkey versus MEF_1 poliomyelitis virus in the mouse. A direct comparison of Helenine and M5-8450 is not possible with these data owing to the differences in dosage employed. The data presented do not justify any conclusion as to the relative effectiveness of the two materials, but seem to be a further indication of their apparent similarity(4,7). Additional studies of the antiviral action of such penicillium filtrates are planned to obtain information regarding their activity and mechanism of action.

Summary. Helenine, an antiviral agent derived from *P. funiculosum,* was found to have marked prophylactic activity in cynomolgus monkeys infected with poliomyelitis virus.

1. Powell, H. M., Culbertson, C. G., McGuire, J. M., Hoehn, M. M., and Baker, L. A., *Antibiotics and Chemotherapy,* 1952, v2, 432.
2. Shope, R. E., *J. Exp. Med.,* 1953, v97, 601.
3. ———, *ibid.,* 1953, v97, 627.
4. Powell, H. M., and Culbertson, C. G., *Antibiotics Annual,* 1953-1954, 147.
5. ———, PROC. SOC. EXP. BIOL. AND MED., 1953, v83, 161.
6. Cochran, K. W., Brown, G. C., and Francis, T., Jr., *ibid.,* 1954, v85, 104.
7. Shope, R. E., *op. cit.,* 639.
8. McClelland, L., personal communication.
9. Mainland, D., and Murray, I. M., *Science,* 1952, v116, 591.
10. Salk, J. E., *Am. J. Pub. Health,* 1955, v45, 575.
11. Smith, P. K., Knox, C. H., and Hollinshead, A. C., *Fed. Proc.,* 1955, v14, 387.

Received April 19, 1956. P.S.E.B.M., 1956, v92.

Protection by Sodium Hydrosulfite Against X-Ray-Induced Mitotic Inhibition in Grasshopper Neuroblast.* (22438)

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It is well known that a reduced oxygen tension during irradiation causes a corresponding reduction in sensitivity to ionizing radiations. The literature on this phenomenon at the cellular level has been reviewed by Giles(1) (chromosome breakage), Muller(2) (mutations), and Gray(3). Recovery from X-ray-induced mitotic inhibition in grasshopper neuroblasts as affected by oxygen tension during irradiation of the intact egg was observed by Gaulden, Nix, and Moshman(4). They found that at 64 r the delay of mitosis was detectably decreased when embryos *in vivo* were irradiated *in vacuo.* At lower doses (3.5 and 8 r) the recovery of mitotic activity appeared to be independent of the oxygen tension

* This work was performed under contract No. W-7405-eng-26 for U. S. Atomic Energy Commission.

around the eggs during irradiation. The small "oxygen effect" at the high dose and the lack of a detectable one at low doses may be due to the inefficiency of a vacuum in removing dissolved oxygen from the fluids of the egg. To eliminate oxygen by a vacuum or by changing the gaseous atmosphere around embryos from which the protecting egg membranes have been removed is not feasible with present technics, because osmotic changes resulting from the evaporation of water interfere with the determination of the X-ray-induced effect. Sodium hydrosulfite is very efficient in protecting bacteria against the lethal effects of X rays(5,6) and in reducing the frequency of radiation-induced chromosome aberrations(7,8). Therefore, sodium hydrosulfite, which readily combines with molecular oxygen in solution(9), was used in the following experiments to remove the oxygen dissolved in the medium.

Materials and methods. Embryos of the grasshopper *Chortophaga viridifasciata* (De Geer) at a stage comparable to 14 days of development at 26°C were dissected in a medium described by Shaw(10). The embryos were pretreated by transfer from the dissecting medium into paraffin chambers filled with medium that contained freshly prepared sodium hydrosulfite. The chambers were sealed with cover glasses to maintain antisepsis and to prevent the diffusion of oxygen from the air into the medium. Within 3-5 minutes after immersion into the pretreatment medium, the embryos were irradiated through the cover glass (0.14 mm) with 8 or 32 r of X rays. A General Electric Maxitron 250 X-ray machine was used at 120 kvp, 25 ma with 3½ mm of Al additional filtration. The target distance averaged 60 cm. The dose rate was 32 r per minute. Within 2 minutes after irradiation the embryos (both irradiated and control) were transferred to a large volume of the dissecting medium to remove the sodium hydrosulfite and its decomposition products. The medium remains isotonic with the embryos when they are kept in a relatively large volume. Hanging-drop cultures were then prepared from the X-rayed and control embryos according to the technics described by Gaulden, Nix, and Moshman(4) and Carlson, Hollaender, and Gaulden(11). The medium used in hanging drops was slightly more dilute than that used for dissection and exposure, in order to compensate for an increase in solute concentration caused by evaporation of water from the medium placed on the cover glass during the preparation of the hanging-drop cultures. The mitotic activity of cultures was determined by counting the number of neuroblasts in mid-mitosis (prometaphase, metaphase, and anaphase) in 6 segments of the embryo (first and second maxillary, first, second, and third thoracic, and first abdominal segments). These segments contain approximately 240 neuroblasts. In all cases, the mitotic activity for each group is the arithmetic mean of the values obtained from 4 embryos. Mitotic ratio is the ratio of the total number of mid-mitotic cells of an irradiated group to those of its control. An analysis of variance† of the raw data was performed to determine whether there was any significant difference between the various groups with respect to the level of mitotic activity. It was found that the use of the new medium(10) and the technic described for handling embryos reduced the fluctuations in mitotic activity between counting periods and between embryos in controls. Thus significant differences could be detected between groups with only 4 embryos per group instead of the 8-16 used by previous workers. Mid-mitotic figures in the cultures were counted at 22-minute intervals. The validity of these values as an index of mitotic activity is discussed by Gaulden and Kokomoor(12). If the duration of mid-mitosis (22 minutes) is not altered by the treatment, each cell that passes through this phase will be counted only once. The treatments used in these experiments caused only a slight prolongation of mid-mitosis before, and occasionally during, the first counting period.

Results. 8 r with no pretreatment. A dose of 8 r of X rays causes about 75% inhibition of mitosis (a mitotic ratio of 0.25) by the 66-minute count (Fig. 1). The mitotic ratio

† The author is indebted to Dr. A. W. Kimball and Mr. George Atta for statistical analysis of the data.

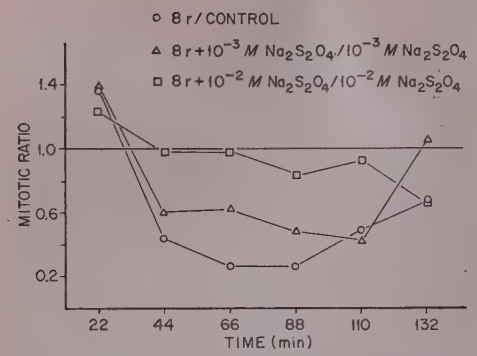

FIG. 1. Effect of concentration of sodium hydrosulfite in pretreatment on mitotic ratio of grasshopper neuroblast cultures.

remains low during the 88-minute count and recovery begins during the 110-minute count. By the time of the 132-minute count the mitotic ratio is 0.67.

Pretreatment with 10^{-3} M sodium hydrosulfite. Short periods of treatment with sodium hydrosulfite arrest mitosis temporarily during the prometaphase-metaphase period. These stages progress again normally if the hydrosulfite is removed within several minutes. After release from blockage by prolonged treatment with sodium hydrosulfite, the rate of progression of the prophase stages into prometaphase and metaphase is considerably slowed. The magnitude of this change in rate is directly dependent on the length of the period of treatment. As the treatment period is lengthened, other abnormalities appear after the release of the cells from the mitotic block; the structure of and rate of formation of the spindle and the condensation of the chromosomes becomes abnormal. A very short treatment with a concentration of 2 x 10^{-2} M sodium hydrosulfite produces the same effects as the prolonged treatment at lower concentrations. The highest usable concentration was 10^{-2} M.

8 r with 10^{-3} M sodium hydrosulfite pretreatment. There is no statistically significant difference in the number of neuroblasts in mid-mitosis of irradiated embryos pretreated with 10^{-3} M sodium hydrosulfite and those which received 8 r alone. Because of the 5-minute pretreatment with sodium hydrosulfite, however, the mitotic activity of

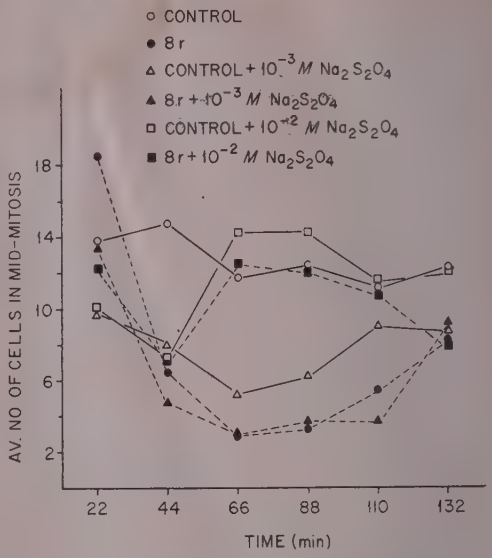

FIG. 2. Effects of X-rays on mitotic activity of grasshopper neuroblast in pretreated and untreated cultures.

the 10^{-3} M hydrosulfite controls is lower than in the untreated controls (Fig. 2). If sodium hydrosulfite at this concentration has both a toxic and a protective effect of equal magnitude, they will compensate for each other and the result will be no change in mitotic activity of irradiated cultures as compared with that of untreated irradiated cultures. Since the mitotic activity of the 10^{-3} M sodium hydrosulfite controls is lower than that of the untreated controls, the mitotic ratio (Fig. 1) of the 8 r plus 10^{-3} M sodium hydrosulfite experiment is higher than in the 8 r experiment with no pretreatment. Comparison of mitotic ratios shows an apparent protective effect of the 10^{-3} M concentration.

8 r with 10^{-2} M sodium hydrosulfite pretreatment. Fig. 2 shows that the depression of mitotic activity of both the X-rayed and of the control cells occurred only during the 44-minute count. This depression, which was due to the toxic effect of the sodium hydrosulfite pretreatment, was less than in the 10^{-3} M experiment because the pretreatment time was reduced to 3-4 minutes. There were no statistically significant differences between the X-ray-treated and the controls in this experiment. This is reflected in the mitotic ratio

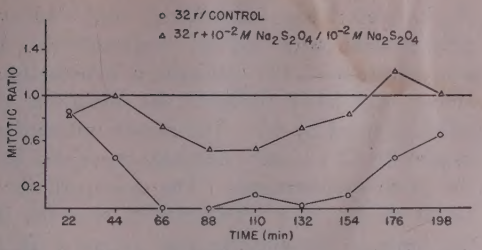

FIG. 3. Effect of pretreatment on mitotic ratio of X-irradiated cultures of grasshopper neuroblasts.

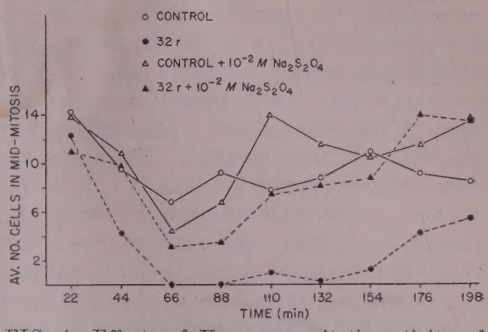

FIG. 4. Effects of X-rays on mitotic activity of grasshopper neuroblasts in untreated and pretreated cultures.

(Fig. 1), which is essentially 1.0 at most of the counts. Thus the pretreatment has almost completely prevented the mitotic inhibition usually caused by 8 r of X rays.

32 r with no pretreatment. A dose of 32 r causes complete inhibition of mitosis by the 66-minute count (Fig. 3) and it remains almost complete through the 132-minute count. By the 154-minute count, recovery begins and the cultures show about 65% of the mitotic activity of the controls (mitotic ratio of 0.65) by the 198-minute count.

32 r with 10^{-2} M sodium hydrosulfite. The mitotic activity of the X-ray-treated cultures is about 70% at the 66-minute count (Fig. 3). However, Fig. 4 shows that both the X-rayed and the control cells had a depressed mitotic activity during the time of the first 3 counts. This inhibition was probably due to the toxicity of the hydrosulfite pretreatment, since the unirradiated hydrosulfite-treated cells show the compensatory rise in mitotic activity during the 88- and 110-minute counts, which is the typical pattern of recovery from a temporary inhibition of mitosis. The mitotic ratio (Fig. 3) shows that the maximum inhibition was only about 50%, and that recovery was complete (when the mitotic ratio equals 1.0) between the 154- and 176-minute counts. There is no statistically significant difference in the shape of the curves of the mitotic ratios for the untreated and the pretreated (Fig. 3). Their heights or levels of mitotic activity, however, are significantly different at the 5% level. Although recovery begins sooner in the cultures receiving the sodium hydrosulfite pretreatment, the rate of return of mitotic activity is apparently the same in cultures that have received the pretreatment as in those that have not. These findings may be interpreted as meaning that the recovery of mitotic activity of the pretreated cultures occurs sooner because there is less radiation-induced mitotic inhibition, and not because the initial rate of recovery is increased.

Discussion. Since sodium hydrosulfite reacts readily with molecular oxygen in solution, the reduction of the radiation effect by sodium hydrosulfite is probably due to a decrease in the quantity of the oxidizing radicals initially produced by the radiations. Gray (3) has pointed out that the presence of oxygen is capable of quadrupling the oxidative yield of X rays through the production of HO_2 radicals, which have the oxidizing equivalent of 3 OH radicals. The reduction of the amount of oxidizing radicals may account for the prevention of the mitotic inhibition induced by 8 r, for at this dose the manifestation of the radiation effect is essentially completely prevented by the hydrosulfite pretreatment. The primary damage responsible for mitotic inhibition at this dose level may therefore be due to the oxidation of intracellular components by HO_2 or similar oxidizing radicals whose formation is dependent on the presence of oxygen during irradiation. At 32 r, since the reduction of the radiation effect by the pretreatment is only partial, the inhibition of mitosis may be only partly caused by the actions of oxidizing radicals.

Gaulden, Nix, and Moshman(4) observed approximately a 50% reduction of mitotic activity with 3.5 r. In the experiments re-

ported here, the reduction of the mitotic activity caused by 32 r in 10^{-2} M sodium hydrosulfite-pretreated cultures is only 50% as contrasted with 100% inhibition in the absence of the pretreatment. This difference is significant at the 5% level. Therefore, the dose-reduction factor is about 8. [The reduction of the total oxidizing power of the radicals is, at most, only a factor of 4. The complete removal of oxygen from the medium by hydrosulfite pretreatment causes a fourfold reduction of the radiation effect as measured by the change in survival of X-irradiated bacteria (5,13)]. At 8 r with the same hydrosulfite pretreatment, the protection is essentially complete. If the dose-reduction factor is 8, then the effective dose is only 1 r. The effect of a dose as small as 1 r would be very difficult to detect. Gaulden, Nix, and Moshman(4) observed only slightly less mitotic delay when intact eggs were irradiated with 64 r *in vacuo,* as in contrast with irradiation in air. A low dose-reduction factor may therefore have been responsible for the lack of detection of a protective effect of the vacuum at the lower doses (3.5 and 8 r). Thus the magnitude of the dose-reduction factor can account for the lack of a detectable protective effect by vacuum at low doses in the work of Gaulden, Nix, and Moshman(4) and for the large protective effect of sodium hydrosulfite pretreatment observed at 8 r presented here.

The dose-reduction factor was, however, actually determined at one dose level (32 r) only. At 8 r, the dose-reduction factor cannot be determined because the residual radiation effect in pretreated cultures is, if present at all, too small to be evaluated. Thus it will require further experiments at other dose levels to be able to decide if the dose-reduction factor is constant with changes in dose. This is of interest because the constancy or lack of constancy of the dose-reduction factor at different doses may offer a clue to the degree of complexity of the primary effect of radiations in causing mitotic inhibition.

Summary. In neuroblast cultures of the embryo of the grasshopper *Chortophaga viridifasciata* (De Geer) protection against X-ray-induced mitotic inhibition is conferred by pretreatment with sodium hydrosulfite. A concentration of 10^{-2} M sodium hydrosulfite almost completely prevents the inhibition of mitosis caused by 8 r. The mitotic inhibition caused by 32 r is only partially prevented by the same pretreatment. The dose-reduction factor at 32 r is 8. The rate of recovery is the same in the cultures that received 32 r and that were also pretreated with 10^{-2} M sodium hydrosulfite as in those that were irradiated but received no pretreatment. The earlier recovery of the pretreated cultures is accounted for on the basis of less demonstrable inhibition of mitotic activity by the radiations. The primary damage responsible for mitotic inhibition at low doses, and at least partially at high doses, may be due to the oxidation of intracellular components by HO_2 or similar oxidizing radicals whose formation is dependent on the presence of oxygen during irradiation.

1. Giles, N. H., in Hollaender, A., *Radiation Biology,* Vol. 1, McGraw-Hill Book Co., Inc., New York, 1954, p713.

2. Muller, H. J., in Hollaender, A., *ibid.,* 475.

3. Gray, L. H., *Brit. J. Radiol.,* 1953, v26, 609.

4. Gaulden, M. E., Nix, M., and Moshman, J., *J. Cellular Comp. Physiol.,* 1953, v41, 451.

5. Burnett, W. T., Jr., Stapleton, G. E., Morse, M. L., and Hollaender, A., PROC. SOC. EXP. BIOL. AND MED., 1951, v77, 635.

6. Burnett, W. T., Jr., Morse, M. L., Burke, A. W., Jr., and Hollaender, A., *J. Bact.,* 1952, v63, 591.

7. Wolff, S., *Genetics,* 1954, v39, 356.

8. Riley, H. P., *Am. J. Botany,* 1955, v42, 765.

9. Lowry, T. M., *Inorganic Chemistry,* 2nd ed. Macmillan & Co., London, 1946, 679.

10. Shaw, E. I., *The Relation of the Composition of the Culture Medium to Mitotic Activity and to X-Ray-Induced Mitotic Inhibition in the Neuroblast of the Grasshopper, Chortophaga viridifasciata* (De Geer). Thesis, Univ. of Tennessee, 1955.

11. Carlson, J. G., Hollaender, A., and Gaulden, M. E., *Science,* 1947, v105, 187.

12. Gaulden, M. E., and Kokomoor, K. L., PROC. SOC. EXP. BIOL. AND MED., 1955, v90, 304.

13. Zelle, M. R., and Hollaender, A., in Hollaender, A., *Radiation Biology,* Vol. II, McGraw-Hill Book Co., Inc., New York, 1954, 365.

Received May 3, 1956. P.S.E.B.M., 1956, v92.